

Single-Reactor Nitritation for High Strength Landfill Leachate Treatment

By

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Abstract

Nitrogen is an important component for our ecosystems, which accounts for 80% of chemical elements in the atmosphere. Ammonia nitrogen is one of the most concerned constituents for the environment. The municipal wastewater contributes the greatest amount of nitrogen into surface water and groundwater in Canada. The landfill leachate could consist of 40-50% of the total amount of nitrogen but with only 1% of the flow in the influent in WWTPs. To meet the more stringent discharge standards for nitrogen in water, highly reliable, stable and cost saving nitrogen removal technologies should be evaluated. The conventional nitrogen removal process nitrification-denitrification has been widely implemented in mainstream treatment. Comparingly, nitritation-denitritation can achieve cost reductions as 25% on aeration and 40% on external carbon demand respectively.

The ammonia-rich landfill leachate with efficient alkalinity is satisfying for the single reactor nitritation process. In this study, an aerobic granular sludge (AGS) reactor was operated in sequencing batch mode at 21°C. The purpose of this thesis is to investigate the feasibility and stability of single reactor nitritation for treating ammonia rich landfill leachate, also analyze the distributions of microbial community and predominant microbes that conduct the nitritation process. After shortening hydraulic retention time (HRT) to 7 hours, the nitritation reactor accomplished the stable ammonia nitrogen removal rate at 99% for more than 100 days. The dominant nitrifying genera was identified as *Nitrosomonas*. The relative abundance showed an increasing trend after long-term operation.

Preface

The work presented in this thesis are my original work and supervised by Professor Yang Liu in the University of Alberta.

Acknowledgment

I would like to express my sincere appreciation to a large number of people. Without the support from them, I could not have achieved the research work presented in this thesis.

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List of Abbreviations

NO_3^-	Nitrate
NO_2^-	Nitrite
NH_4^+	Ammonium
AMO	Ammonia Monooxygenase
AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
COD	Chemical oxygen demand
Comammox	Complete ammonia oxidation
CSTR	Completely stirred tank reactor
DNRA	Dissimilatory nitrate reduction to ammonia
DO	Dissolved oxygen
DGGE	Denaturing gradient gel electrophoresis
FA	Free ammonia
FH	Free hydroxylamine
FNA	Free nitrous acid
HAO	Hydroxylamine Oxidoreductase
HNO_3	Nitric acid
HRT	Hydraulic retention time
HZO	Hydrazine oxidase
IFAS	Integrated fixed film and activated sludge
MBR	Membrane bioreactor
MBBR	Moving bed bioreactor
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids

NA	Nitrite accumulation
Nar	Nitrate reductase
NH ₃	Ammonia
Nr	Reactive nitrogen
Nir	Nitrite reductase
NO	Nitric oxide
NOB	Nitrite Oxidizing Bacteria
Nor	Nitrite Oxidoreductase
Nos	Nitrous Oxide reductase
NO _x	Nitrogen oxides
SBR	Sequencing Batch Reactor
SRT	Solid retention time
SVI	Sludge volume index
TIN	Total inorganic nitrogen
TKN	Total Kjeldahl nitrogen
WWTPs	Wastewater treatment plant

Chapter 1 – Introduction

1.1 Nitrogen impact and wastewater discharge regulations

Nitrogen (N) accounts for 78.1% of Earth's atmosphere and plays a critical role in all kinds of life. Through the fixation process, either by nature or in an anthropogenic manner, the unreacted nitrogen (N_2) can be converted into the reactive form of Nr and consumed by the living organism (Stevens, 2019). Ammonia is one of the most commonly detected poisonous materials from various waste sources, including fertilizers, wastewater from irrigation, and animal wastes (Sugawara & Nikaido, 2014). The adverse effects created by ammonia are eutrophication, endangering aquatic life and humankind health, and a noisome odor (Alonso & Camargo, 2015). Furthermore, water quality could be degraded by the disposal of ammonia-entrained effluent (Panagopoulos, 2022a, 2022b; Panagopoulos & Haralambous, 2020). It is important to get ammonia removed at an acceptable level from wastewater to protect our environment. In Canada, New Zealand, China, and Australia, the related ammonia quality criteria in water are 4.82, 2.18, 12, and 2.18 mg/L, respectively (standard conditions at pH 6.5~9.0 and temperatures at 5~30°C) (Yan et al., 2020). Under the license-to-operate requested by Alberta Environment, wastewater treatment plant's (WWTP) discharge limit for ammonia-nitrogen was 5 mg/L in the summer season and 10 mg/L in the winter season. In light of the high NH_3-N (10~ 60mg/L) and total Kjeldahl nitrogen (TKN) (35~70 mg/L) concentrations inside the influent stream feeding to the WWTP, reliable nitrogen removal strategies need to be in place prior to the discharge process.

Landfill leachate is a complex, dark-colored effluent generated from landfills. It contains high levels of organic material, ammonia, dissolved solids, heavy metals, and xenobiotic organic compounds (Renou et al., 2008). The characteristics of the landfill leachate may differ depending on the amount and morphology of the deposited waste, the environmental conditions in terms of

temperature, rainfall, and the age of the landfill (J. Li et al., 2019). The treatment of leachate is mainly to consider two elements: volumetric flow rate and its composition. Before releasing into water bodies, it is critical to remove COD, BOD, and ammonium levels (Chua K H et al., 2011). The treatment of leachate can be classified into two groups, including conventional and advanced treatment. Conventional treatment can further be divided into three sub-groups. First is the leachate transfer as well as recycling or reinjection to landfill cells (Ferraz F M et. al, 2016). Second are the biological degradation processes, which include aerobic and anaerobic processes. The third sub-group are the physiochemical processes such as adsorption, air stripping, coagulation/ flocculation, advanced oxidation, chemical precipitation, etc.

1.2 Ammonia removal by AGS

CAS (Conventional activated sludge) has been developed for more than one century and is still utilized universally due to its low cost, simplicity, and operation stability (Maltos et al., 2020). However, the limitations of CAS are obvious in terms of low SRT and the elevated cost of treating sludge. Thereafter, some innovative CAS processes were developed to address the above issues. In this study, we will focus on discussing AGS's capability of treating ammonia from wastewater. AGS (Aerobic granular sludge) has proven capabilities of removing organic substances and nutrients simultaneously with smaller reactors and utilizing less energy. Over 40 full-scale plants are in operation around the world after this technology was established 35 years ago. The major bottlenecks of preventing AGS's implementations universally, including slow granulation, long-term startup, granular stability issues, and biomaterial recovery from waste sludge, are not fully overcome yet (S Mardones et al., 2019) .

Various reactor types are practical to achieve ammonia removal. The SBR reactor fed with

operated synthetic wastewater and operated in anammox and partial denitrification mode. It achieved a 94% total nitrogen removal rate (Du et al., 2015). Similarly, using partial nitrification and anammox processes, the plug-flow AGS pilot-scale reactor accomplished 46% nitrogen removal at the A-stage of Dokhaven WWTP in Rotterdam (Lotti et al., 2015). Recently, CFR (Continuous flow reactor)- AGS received lots of attention, and many laboratory-scale studies were implemented. More research is expected to be completed in this area to approve the CFR-AGS feasibility and stability before the full-scale operation takes place in the future (Rosa-Masegosa et al., 2021).

1.3 Research objectives

In this study, a column aerobic granular reactor was operated in sequencing batch mode to perform a single reactor nitrification reaction for ammonia-rich landfill leachate treatment. The objectives of this study are to:

1. Evaluate the long-term feasibility and stability of single reactor nitrification for treating ammonia-rich landfill leachate;
2. Examine the nitrogen removal kinetics within aerobic granular sludge;
3. Demonstrate the distributions of microbial communities in an aerobic granular sludge system after a long period of operation.

1.4 Thesis structure

Chapter 1 introduces the relevant background information about this project and explains the scope, the current research gap, the objectives, and the structure of this thesis. Chapter 2, a literature review, describes conventional and advanced nitrogen removal processes and also covers microorganisms related to nitrogen removal. As to Chapter 3, methods of testing the water

chemistry and the dynamics of the microbial community inside the reactor are discussed; furthermore, the performance of reactors is explained in detail. Chapter 4 shows the nitrification results after 9 months of operation. Within this, reactor performance, kinetic studies, and microbial dynamics about the single reactor SBR operation are interpreted. Chapter 5 summarizes this study and provides recommendations for enhancing reactor performance in future studies.

Chapter 2 – Literature Review

2.1 Overview of nitrogen and ammonia in the environment

The reactive nitrogen (Nr) circulation rate has been doubled by humans along with the economic growth, which brings about dramatic changes and threats to climate, food and energy security, ecosystem balance, and eventually human health. The forms of Nr are made up of NH₃, Nitrate (NO₃⁻), NO_x, N₂O, amines, and organic nitrogen, excluding N₂ (Erismann et al., 2011). Ammonia is a rising concern globally; if not treated properly, it can lead to adverse effects on aquatic ecosystems and produce harm to forests, crops, and other vegetation. The more stringent discharge limits are faced by municipal wastewater plants. For example, China's limits are 5 mg/L ammonium, 15 mg/L total nitrogen (GB18918-2002) (Ritter et al., 2002). It is paramount and urgent to treat ammonia below acceptable levels.

The commonly employed technologies to reduce ammonia concentrations are physicochemical and biological processes. Each process has its advantages and disadvantages. As to physicochemical methods, ion exchange, breakpoint chlorination, adsorption, or electrical oxidation are widely applied. Comparingly, biological processes are more universally operated in wastewater treatment plants thanks to their cost efficiency and resilience to environmental changes (Ren et al., 2022). Aerobic ammonia oxidizers and anaerobic ammonia oxidizers are two groups of bacteria responsible for ammonia reduction in wastewater. In regard to the specific biological process, except conventional nitrification and denitrification techniques, some emerging methods are gradually implemented in WWTPs, for instance, partial nitrification (nitritation), anaerobic ammonia oxidation (ANAMMOX), completely autotrophic nitrogen removal over nitrite (CANON), single reactor high activity ammonia removal over nitrite (SHARON), and Oxygen-limited nitrification and denitrification (OLAND) (Schmidt et al., 2003).

2.2 Physiochemical and biological nitrogen removal

2.2.1 Stripping

The concept of the ammonia stripping process is, based on the mass transfer principle, to allow wastewater in contact with air, then the ammonia gas contained in the wastewater gets removed (Kinidi et al., 2018). The pH and temperature of the wastewater will determine the forms of ammonia, either in the gas phase or in the ion phase. When the pH is high, ammonia gas as the governing phase promotes the ammonia stripping performance; therefore, lime is commonly supplemented prior to the stripping process (Wang et al., 2007). The benefits of applying an ammonia stripping process are ease of operation, cost efficiency, and the potential recovery of stripped ammonia from wastewater (Ozturk et al., 2003). Nonetheless, pilot-scale studies and in-depth economic assessments are prerequisites for the large-scale operation in practice (Kinidi et al., 2018).

2.2.2 Ion exchange

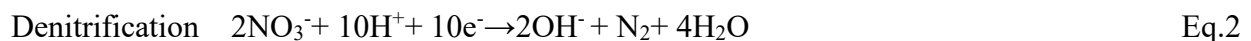
Ion exchange is originally utilized for water softening, which removes calcium and magnesium ions from water. The concept is to substitute charged ions into the targeting dissolved ions, and it is a reversibly chemical reaction with exothermicity (Karri et al., 2018). Eventually, this technology extended its applications to the wastewater treatment industry, specifically, extensive research on ammonia removal at laboratory or pilot scales. The performance factors include hydraulic retention time, pH, temperature, and chemical and biological regeneration capacity for ammonium-saturated zeolites (Hedström, 2001). Even though the ion exchange's capability to tackle a wide range of concentrations of ammonia and temperature resilience, the significant operation cost associated with chemical reagent regeneration are the major constraints preventing its widespread applications (Imchuen et al., 2016).

2.2.3 Membrane

The pressure gradient drives membrane filtration technology. It stops solids and dissolved elements from passing through the membrane (Karri et al., 2018). The most commonly utilized membrane processes for treating wastewater are reverse osmosis, ultrafiltration, microfiltration, and nanofiltration (Ezugbe & Rathilal, 2020). Europe has been leading the way in using membrane contactors since 2002. They use this technology to remove ammonia from wastewater on a large scale (Karri et al., 2018). There are many advantages of using membrane contactor in comparison to conventional ammonia separation processes, such as faster ammonia removal rate, insubstantial folding and foaming, reduced capital cost, and easy scale-up (Ashrafizadeh & Khorasani, 2010; Gabelman & Hwang, 1999; Hasanoğlu et al., 2010). Nevertheless, fouling problems and short lifespans caused by recurrent maintenance are common issues and need more research to overcome (Karri et al., 2018).

2.2.4 Nitrification and denitrification

The two-step biological process of ammonia oxidation is called nitrification. One form of autotrophic bacteria converts ammonia to nitrite (NO₂-N) in the first stage, and another type of autotrophic bacteria converts nitrite to nitrate (NO₃-N) in the second stage. In contrast, the biological process of reducing nitrate or nitrite to nitrogen gas by heterotrophic bacteria is known as denitrification (Metcalf & Eddy, 2014). The nitrification and denitrification process equations are seen from below Eq.1 and Eq.2. (Jenicek et al., 2004):



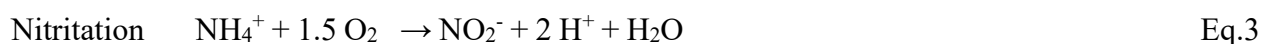
According to Metcalf and Eddy (2014), dissolved oxygen concentrations, pH, toxicity, metals, and

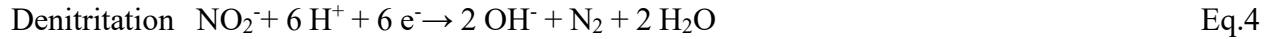
unionized ammonia are among the environmental elements that have an impact on the nitrification process. Based on the above equation 1, the oxygen needed for complete oxidation of ammonia via nitrification is 4.57 g O₂/g NH₄-N oxidized. Regarding the alkalinity consumption, nitrification requires around 7.14 g of Alkalinity as CaCO₃ consumed per g of NH₄-N oxidized (Metcalf & Eddy, 2014). Controlling DO concentration is essential in AGS process for efficient nitrogen removal. DO level should be sufficient enough for ensuring complete nitrification. Meanwhile, DO value needs to be in proper low level to achieve simultaneous denitrification (De Kreuk, Heijnen, et al., 2005).

Denitrification environmental factors are about pH and external carbon source. Organic carbon is recognized as the essential element to achieve any denitrification process (Pochna & Keller, 1999). For most of the municipal wastewater, a TCOD:TKN ratio as 7 is identified as a key factor in performing complete denitrification (Takashi Asano; Paul Bishop, 1998). However, this TCOD/TKN ratio needs to be increased to at least 9 to obtain nitrogen and phosphorous removal simultaneously (Goronszy, 1992). In regarding to the denitrification efficiencies by the most commonly carbon sources, acetate ranks higher than methanol, with glucose as the lowest status (Gerber et al., 1986; Tam et al., 1992).

2.2.5 Nitritation and denitritation

Even though nitrification and denitrification are widely used in WWTTs, however, nitrogen removal via nitrite pathway has many advantages. Nitritation is the biological process to only oxidize ammonia to nitrite (NO₂-N). As to the denitritation, it is the biological pathway of reducing nitrite to nitrogen gas (Metcalf & Eddy, 2014).





Carbon source is the most essential factor for complete denitrification. 3.7 g COD/ g N is required for complete denitrification, and 2.3 g COD/g N as the value needed for complete denitrification respectively (Metcalf & Eddy, 2014).

Seen from the above equation 3 and 4, the benefits of nitrogen removal through nitrite pathway verses nitrate are obvious: 1) quicker reaction time; 2) roughly 25% less oxygen required for nitrification comparing to nitrification; 3) around 40% lower organic substrate demand for denitrification as compared to denitrification (Jenicek et al., 2004).

2.2.6 Anaerobic ammonia oxidation (Anammox)

Since the anammox bacteria was discovered in 1995 by Mulder's group, a new generation of anaerobic digestion started. Mulder et al. (1995) determined the anammox process equation for anammox as below equation 5. He also confirmed that ammonium could directly be oxidized to nitrogen gas with nitrite as the electron acceptor under anoxic conditions.



Up to now, six anammox genera and more than 20 species have been identified. From wastewater treatment plants and freshwater environments, *Candidatus Brocadia* (*C. Brocadia*), *C. Kuenenia*, *C. Anammoxoglobus*, *C. Jettenia*, *C. Anammoxomicrobium* were identified, and *C. Scalindua* was discovered from marine environments (Jetten et al., 2009; Khramenkov et al., 2013). Later on, the following researchers proved the three benefits of anammox bacteria compared to the conventional nitrification/denitrification system, which are: 1) a higher total nitrogen removal rate, 2) energy savings due to only anoxic reactions required, 3) a small footprint in the application (Z. Li et al., 2014). However, the limitation of the Anammox process for treating real wastewater is the extremely long startup time because of the Anammox bacteria's slow growth rate and strict

metabolic environments (Ibrahim et al., 2016). Starting in the 2010s, the partial nitrification/anammox (PN/A) process gained tremendous attention and showed promising results in diversified-scale applications, even in challenging conditions due to low nitrogen loading rates and low process temperatures (10~15 °C) (Cao et al., 2017).

2.2.7 Dissimilatory nitrate reduction to ammonium (DNRA)

DNRA is a well-known process as nitrogen transformation, which is similar to denitrification but with the distinction of nitrate being reduced to ammonia instead of nitrogen gas. It can be considered the converse pathway to Comammox (Kraft et al., 2011). DNRA bacteria are widespread in most ecosystems. Domestic wastewater treatment plants use the cytochrome c nitrite reductase (*nrfA*) gene to identify them (S. Wang et al., 2020). The abundance and reaction rate of DNRA were proven to be lower than denitrification (S. Wang et al., 2020). DNRA has gained a huge focus following the detection of metabolically multi-functioning bacteria. More research is expected to be implemented in the future (Xi et al., 2022).

2.3 Microbes

2.3.1 Nitrifying Microorganisms

There are four types of bacteria that can convert ammonium to nitrite or nitrate. These bacteria are ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), ammonia-oxidizing archaea (AOA), and complete ammonium-oxidation microorganisms (Comammox) (Koch et al., 2019).

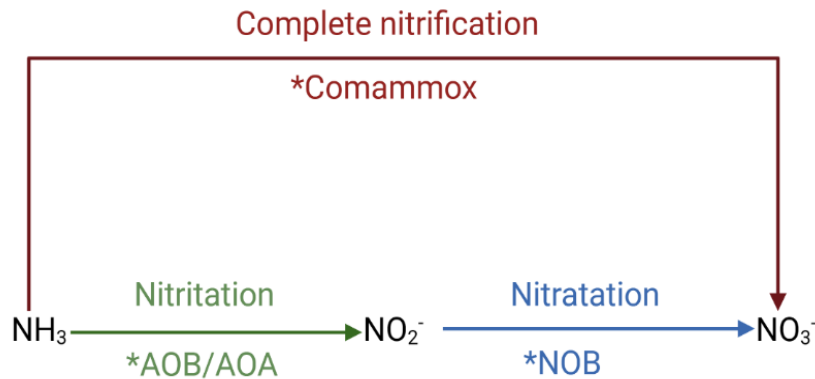


Fig.1 Overview of different microorganisms engaged in ammonia oxidation. AOB is ammonia oxidizing bacteria, AOA is ammonia oxidizing archaea, NOB is nitrite oxidizing bacteria, Comammox represents Complete ammonia oxidizer. (Drawn by biorender.com)

(i) Ammonia oxidizing bacteria (AOB)

The first AOB was isolated back in 1890, and then there were a number of studies implemented to comprehend generic diversity (Koops & Pommerening-Roser, 2006; Purkhold et al., 2000). There were a total of five genera AOB confirmed as Proteobacteria class. Four of them fall into the β -Proteobacteria subclass, containing *Nitrosomonas*, *Nitrospira*, *Nitrosovibrio*, and *Nitrosolobus*; with the last genera *Nitrosococcus* related into γ -Proteobacteria subclass (Soliman & Eldyasti, 2018). Geets et al. (2006) reviewed and considered *Nitrosomonas* as the predominant nitrifying microorganism in wastewater treatment systems, with only several pieces of research identifying *Nitrosospira*-related strains' dominant status. Based on the former studies, *Nitrosomonas* and *Nitrosospira* are the most essential microorganisms in the activated sludge systems (H. D. Park & Noguera, 2004; Purkhold et al., 2000). In these two genera, *Nitrosomonas* is recognized as a predominant status in many bioreactors (Limpiyakorn et al., 2011; H. D. Park & Noguera, 2004; Wells et al., 2009). Oshiteru Aoi, et al. (2000) pointed out that reactor types, influent

characteristics, and operation conditions are determined factors about AOB ecology and predominance circumstances in engineering systems. As to the reactor type, at SBR treating an extremely concentrated nitrogen leachate process, *Nitrosomonas europaea*, and *Nitrosomonas eutropha* were detected as dominant bacteria by employing DGGE fingerprinting and PCR amplification (Gabarró et al., 2012).

(ii) Nitrite oxidizing bacteria (NOB)

The recognized nitrite oxidizers belong to seven genera *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospira*, *Nitrospina*, *Nitrolancea*, *Candidatus Nitromaritima* (Daims et al., 2016). NOB is very challenging to nurture, and the isolation of pure culture could take up to twelve years (Lebedeva et al., 2008). *Nitrospira* lineages I and II are identified as vital members in WWTPs (Daims et al., 2001; Juretschko et al., 1998; Kruse et al., 2013). Recently, *Nitrotoga* has been detected as another vital NOB division in engineering systems (Alawi et al., 2009; Hüpeden et al., 2016; Lücker et al., 2015; Saunders et al., 2016). Intriguingly, *Nitrospira* and *Nitrotoga* are found in coexistence in some WWTPs (Alawi et al., 2009; Lücker et al., 2015). The relatively low temperature (10 ~17 °C) range proved to be in favor of *Nitrotoga* strains enrichment in a WWTP. As to *Nitrotoga*, it can be cultivated in a wider temperature range from 10~28 °C (Alawi et al., 2009).

(iii) The AOB and NOB distributions

Researchers found two factors impacting AOB and NOB microbial communities switching and respective kinetics. They are alterations of environments and applied operating circumstances (Cao et al., 2017). Temperature has a substantial impact on diversity and kinetics (primarily on μ_{max}). For instance, in the side stream partial nitrification/ anammox (PN/A) process, the widespread implementation of minimum sludge retention time (SRT) plus the temperature verses AOB and

NOB correlations are (Hellingsa, 1998): μ_{\max} .AOB is greater than the value of NOB when the temperature exceeds 20 °C, whereas the μ_{\max} .NOB is greater than the rate of AOB at low temperatures below 20 °C situations (Hunik, 1993). DO also has a remarkable influence on the diversity and kinetics of AOB and NOB (Bellucci et al., 2011; G. Liu & Wang, 2013; Park & Noguera, 2008). Specifically, after operating the reactor for half a year in high-DO circumstances, the *Nitrospira* community transformed from group 1 to group 2, and at the same time in the low-DO reactor, no major transformation was detected in terms of the group 1 *Nitrospira* community (Park & Noguera, 2008). It was recorded that with longer than one-year low-DO operation, the dominant AOB remained as *N. europaea/eutropha*. However, the population growth for *Nitrospira-like* NOB was greater than that for *Nitrobacter-like* NOB on account of the decreased nitrifier endogenous decay rate and low-DO operating conditions (G. Liu & Wang, 2013).

(iv) Enriching AOB over NOB

Several factors have been proven to be effective in inhibiting NOB and leading NOB washout from the systems. These include high pH, high free ammonia (FA) concentrations, free nitrous acid (FNA), low dissolved oxygen (DO), and short SRT in combination with high temperature (Lemaire, Marcelino, et al., 2008). By stepwise increasing influent ammonia concentrations, nitrification was successfully achieved as the result of a major part of AOB distributed at the granular surface while almost no NOB was present. It was hypothesized that FA inhibition and finite DO diffusion capacity within granules resulted in the stable operation of the nitrification process (Shi et al., 2011).

(v) Ammonia oxidizing archaea (AOA)

In 2005, the first AOA was isolated and captured from a marine aquarium tank, also entitled

Candidatus Nitrosopumilus maritimus; furthermore, AOA was confirmed with the ammonia oxidation capacity (Könneke et al., 2005). Thereafter, more proofs were identified that AOA was present in the CAS wastewater treatment systems (Park et al., 2006; Wells et al., 2009; T. Zhang et al., 2011; Limpiyakorn et al., 2011). There are some major differences between AOA and AOB; the first one is that no HAO homologues are identified in the AOA genome (Simon & Klotz, 2013; Walker et al., 2010); the second disagreement comes from the differing strain trait of hydroxylamine accumulation of AOA versus AOB; the third deviation is the way in which AOB and AOA fix inorganic carbon and utilize organic carbon, through which AOB employs the Calvin-Benson-Bassham (Calvin) cycle of carbon fixation (Hatzenpichler, 2012). Researchers found out that AOA was predominate over AOB in a membrane bioreactor system (MBR) at elevated mixed liquor concentrations greater than 8,000 mg/L and decreased DO concentrations lower than 0.20 mg/L, while this prevailing trend declined in chilling temperature circumstances (Giraldo et al., 2012a, 2012b). Further studies showed that AOA could be more practically enriched and cultured in extreme situations, even though more convincing investigations are expected to be conducted shortly (Yin et al., 2018). To my best knowledge, it is not fully known how AOA and AOB distribute in various wastewater treatment systems; the factors in play could be water chemistry such as ammonia concentrations, organic loading, process operation conditions in terms of dissolved oxygen (DO) concentrations, and temperature (Sinthusith et al., 2015).

2.3.2 Denitrifying Microorganisms

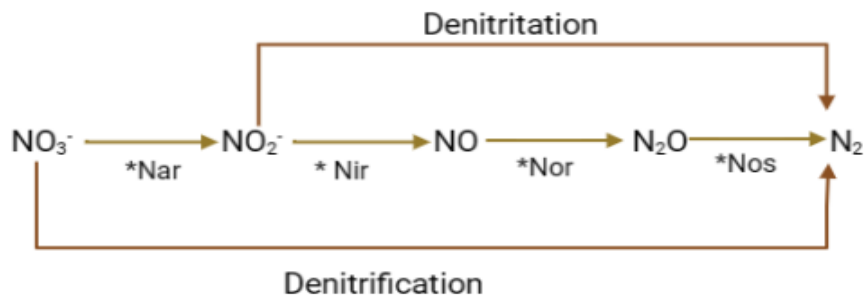


Fig.2 Enzymes involved in complete denitrification and denitrification pathways. Nar is nitrate reductase, Nir is nitrite reductase, Nor is nitric oxide reductase, and Nos is nitrous oxide reductase.

(i) Heterotrophic denitrifying bacteria

The denitrification phenomenon is discovered extensively in bacterial and archaeal areas. Generally, the denitrifying microorganisms in wastewater treatment systems are not as diverse as the ones in soil (Throbäck, 2006). After isolating from denitrifying reactors, bacteria strains closely linked to *Hyphomicrobium*, *Paracoccus*, *Pseudomonas*, and *Comamonas* spp. In Proteobacteria (Chakravarthy et al., 2011; Gumaelius et al., 2001; Martineau et al., 2013; Su et al., 2001). The predominance of denitrifying communities between different wastewater treatment plants rely on influent properties, process layouts, and operating parameters (Lu et al., 2014). There are five groups based on denitrifying activities. The first group is complete denitrifiers and can reduce nitrate and nitrite to N_2 . Examples include *Hyphomicrobium* spp. (Sperl & Hoare1, 1971). The second group is exclusive nitrite reducers, which are only competent to reduce nitrite to N_2 , but not for nitrate. The third group is incomplete denitrifiers that are qualified to reduce either nitrate or nitrite to nitrogen oxide, rather than N_2 . Examples include *Methyloversatilis* spp. (Lu et al., 2012). The fourth group is incomplete nitrite reducers and capable of reducing nitrite or nitrate to nitrogen oxide. One example is *Pseudomonas* spp. (Somsak Vangnai & Klein, 1974). The last group is non-denitrifiers that are not competent for reducing either nitrate or nitrite.

(ii) Autotrophic denitrifying bacteria

The denitrifying microorganisms can take energy from various sources, such as organic, inorganic, or both. Accordingly, these microbes are categorized as heterotrophic, autotrophic, or mixotrophic (Di Capua et al., 2019). Some existing identified autotrophic denitrifying bacteria include *Thiobacillus denitrificans* reducing sulfur compounds (Bock et al., 1995), *Nitrosomonas eutropha*, *Nitrosomonas europaea* and *Nitrosolobus multiformis* (Bock et al., 1995; Poth & Focht1, 1985; Schmidt et al., 2003; Zart & Bock, 1998), *Paracoccus denitrificans*, *Paracoccus ferrooxidans*, *P. pantotrophus* and *P. versutus* oxidizing zero-valance iron and Fe (II) (Kielemoes et al., 2000; Kumaraswamy et al., 2006). However, the reaction rate of *Nitrosomonas* in oxidizing ammonia is so slow that it brings a minor operational impact on activated sludge treatment processes (Littleton et al., 2002). Autotrophic denitrification (AuDen) mostly occurs in attached growth systems. These systems include membrane biofilm reactors (MBfR), biofilters, and fluidized bed reactors (FBRs). They are designed to support high concentrations of biomass growth (Di Capua et al., 2015).

(iii) Aerobic denitrifying and heterotrophic nitrifying (HD-AD) bacteria

The first HD-AD microorganism was detected in 1988 (Robertson et al., 1988). Up to today, several HD-AD microorganisms such as *Alcaligenes faecalis* (Joo et al., 2005), *Acinetobacter* sp. (S. Chen et al., 2019), *Cupriavidus* sp. (Sun et al., 2016) were effectively isolated from diverse environments, for instance landfill leachate (Q. Chen & Ni, 2011), saline wastewater (H. Wang et al., 2020), pharmaceutical (J. R. Yang et al., 2019). Initially, the studies focused on separating different types of HD-AD bacteria. Later, the focus shifted to optimizing nitrogen removal in controlled operations (C. Li et al., 2015; J. Zhang et al., 2011). Despite the potential of HD-AD to remove carbon, nitrogen, or phosphorous from wastewater, it is not effective enough to treat real

wastewater (L. Yang et al., 2019). Engineers can couple HD-AD along with other processes to deal with complex real wastewater when designing a robust and resilient system (Xi et al., 2022).

2.4 Biological treatment processes

Comparing to physiochemical methods, biological treatment is privileged to be a proper way due to the lower cost when adopting various microorganisms produced by nature (Karri et al., 2018).

2.4.1 CAS (Conventional activated sludge)

In 1914, Ardern and Lockett developed the conventional activated sludge process (CAS) at the Davyhulme sewage works in Manchester, England. They returned the retained solids to the system, and reduced the purification time from two weeks to 24 hours (Jenkins, 2014; Metcalf & Eddy, 2014). The first CAS full-scale continuous-flow wastewater plant was in operation in 1916. Since then, CAS has been employed universally as a biological treatment for municipal and industrial wastewater (Nelson, 2016). The CAS process for wastewater treatment converts organic matter to biomass and carbon dioxide (Gu et al., 2017). The aeration tank and the following settling tank (or clarifier) are essential for accomplishing these functions. When wastewater mixes with microbes at aeration conditions, it forms mixed liquor suspended solids (MLSS) or mixed liquor volatile suspended solids (MLVSS). The mixed liquor then settles and thickens in the downstream tank (Nelson, 2016). The settled biomass, known as “activated sludge”, contains microorganisms to improve wastewater treatment performance. It needs to be returned back to the aeration tank to continue breaking down organic substances in the influent. To prevent solids built-up, it is common to remove the thickened solids daily or regularly (Metcalf & Eddy, 2014).

Although CAS has been used with advanced engineering experience, it does have cost and treating efficiency challenges. The system produces a lot of waste sludge, which costs 50~60 % to treat

(Campos et al., 2009). One constraint is that most WWTPs only partially degrade the micropollutants. These WWTPs are designed to remove organic matter and low-strength nitrogen or phosphorus (Nelson, 2016). Some of the solutions promoted by researchers included combining aerated reactors with non-aerated ones, connecting the reactors with recirculation lines, and building sludge recycling lines from settling tanks to bioreactors (Hreiz et al., 2015).

2.4.2 Membrane bioreactor (MBR)

MBR is the integrated process of biological methods (aerobic and anaerobic) with membrane filtration. The first MBR was built by Smith et al. In 1969, they treated manufacturing wastewater using an ultrafiltration membrane instead of a sedimentation tank. This helped them achieve desirable effluent quality. However, excessive energy cost and membrane fouling prevented this technology from being applied commonly (Radjenović et al., 2008). In 1989, Yamamoto's research team made two important innovations. They installed a hollow-fiber membrane inside an aeration tank. They also utilized suction pressure instead of a pressurized pump. These changes made the immersed-membrane layout practical (Yamamoto et al., 1989). Afterward, more research was conducted, and MBR plants were developed and used widely (Al-Asheh et al., 2021). There are two types of membranes set up based on their locations. Side-streamed MBR is the layout with the membrane external, and submerged MBR immerses the membrane directly into the reactor (Ladewig et al., 2017). Hollow fiber, tubular, and flat sheet are the three widely used types of membranes (Al-Asheh et al., 2021).

The MBR system can achieve high MLSS concentrations by separating and trapping biomass with the membrane. However, the studies found that the permeability decreased when MLSS exceeded 10,000 mg/L (Trussell et al., 2007). Furthermore, the viscosity increase made filterability worse (Wu & Huang, 2009). The optimum MLSS concentrations are in the 8,000-12,000 mg/L range

(Al-Asheh et al., 2021). MBR has several advantages compared to CAS. These include higher SRT because of the lower HRT, better effluent quality, and more efficient sludge separation (Al-Asheh et al., 2021; Metcalf & Eddy, 2014). However, the obstacles to the MBR process are associated with the membrane fouling problem, the complexity of membrane maintenance, the cleaning procedure, and higher operation costs (Al-Asheh et al., 2021). Since the 2000s, MBR has made promising progress in treating typical pollutants, pathogens, and other emerging pollutants from wastewater in full-scale applications. However, we must solve these problems before we can use MBR widely. To control fouling, we can optimize hydrodynamic situations like membrane modules and tank configurations. We can also investigate novel membrane materials to make them more hydrophilic. Cost reduction can be achieved through bioreactor combination methods. Another option is to use a hybrid or integrated MBR with other processes (Al-Asheh et al., 2021).

2.4.3 Biofilm based processes (MBBR)

The biofilm process has been in focus due to the higher loading and decent removal efficiency of a compact system. Moving bed membrane reactor is one of the most commonly utilized processes of biofilm types reactors (Dezotti et al., 2017). The MBBR process was facilitated in Norway in the late 1980s by the incorporations set up between the Norwegian company AnoxKaldnes and one water treatment research group from the NTNU-Norwegian University of Science and Technology (Rodgers & Zhan, 2003; Odegaard et al., 1999). By 2014, there were around 1,200 MBBR facilities operating in 50 countries (Biswas et al., 2014). This nitrogen removal process is versatile and reliable. It requires less maintenance for biofilm control and flushing out solids (Metcalf & Eddy, 2014). MBBR can be identified as the combined process of biofilter and activated sludge. Biocarriers are freely moving carriers (mostly small plastic hollow cylinders) for biomass to attach and grow into inside biofilm, which consumes the dissolved pollutants in the

wastewater (Leiknes & Ødegaard, 2007). MBBR has proven treatment efficiency in terms of BOD, COD, total phosphorus, and total nitrogen removal with up to 96~99% for municipal and industrial wastewater at 2~6 h HRT operation conditions (Madan et al., 2022). The key factors affecting MBBR performance are biocarriers, filling fraction, and dissolved oxygen. The most widely used carrier materials are high-density polyethylene (HDPE), polypropylene (PP), or polyethylene (PE) with a lighter density than water (Ødegaard et al., 2000). The ideal carrier is classified as insoluble, lighter-density, high effective specific surface area containing inert, non-toxic habitats for microorganisms to grow (Dias et al., 2018; Singh Chaudhary et al., 2003). One of the prime merits of MBBR is that the process can be adjusted to be aerobic, anoxic, or anaerobic by retrofitting biocarriers (Madan et al., 2022).

The filling fraction is the ratio of the added carrier volume over the total reactor volume. It is predetermined to tune the filling fraction with MBBR for treating purposes, according to proven experimental data showing 50% ~ 60% suitable for organic matter removal and 30% ~ 40% functional for nutrient removal (Madan et al., 2022). DO level is typically over 2 mg/L. The proper design of the reactor should secure a constant air supply, move the biocarriers continuously, and not shear off the prime biofilm from the attaching media (Dezotti et al., 2017).

2.4.4 Integrated fixed-film activated sludge (IFAS)

IFAS is a hybrid process by adding attached media into the CAS system to facilitate biomass growth. The media can be fixed or free-floating, indicating IFAS contains both biofilm and flocs (Albizuri et al., 2009; Mahendran et al., 2012). These combined structures enable IFAS to reinforce nutrient removals due to the promotion of slow-growth nitrifying bacteria in the biofilm. Compared to CAS and MBR, IFAS illustrates multiple advantages, including higher organic and nitrogen removal performance, a smaller footprint, complete nitrification, and longer SRT. Moreover, by

simply integrating carrier media, the existing CAS system can be upgraded to IFAS (Singh et al., 2017). The three essential affecting parameters are carriers' media filling ratio, operation conditions, and biology. It is very important to define the right carrier media. This includes material, type, and filling ratio (Hooshyari et al., 2009; Sriwiryarat & Randall, 2005). Recently, combining IFAS with a membrane system has shown success for diverse microbes and resistance to toxins (Eslami et al., 2018; Marbelia et al., 2014). However, more research about the IFAS-MBR integration system is required to get a deeper insight into membrane fouling control and the microbial community (Waqas et al., 2020). IFAS is a promising process for treating municipal and ammonia-rich industrial wastewater. The biofilm inside the IFAS system promotes the stable growth of AOB, AnAOB, NOB, and anammox bacteria. Anaerobic MBBR and IFAS-SBR have the potential for energy recovery. More studies are expected to improve the operation conditions and provide a deeper understanding of the A-B process (Gu et al., 2017).

2.4.5 AGS

In the late 1990s, AGS was found as a new type of aggregated flocs during aerobic wastewater treatment processes (Mishima & Nakamura, 1991; Shin et al., 1992). They are characterized by the remarkably greater size and compact structure (Morgenroth et al., 1997), even though these granules are associated with the identical categories of microorganisms (Winkler et al., 2013).

AGS process is an essential substitute for CAS (Franca et al., 2018). The sludge volume index (SVI) was identified as the indicator to distinguish AGS versus CAS. Specifically, the value of SVI_{30} / SVI_5 of AGS is above 0.8 (Derlon et al., 2016; Schwarzenbeck et al., 2005a). Generally, AGS particle size is greater than 0.2 mm, ensuring fast settling (De Kreuk et al., 2007). In practice, SVI_{30} ranges from 30~60 mL/g TSS, much lower than the CAS value of around 100 mL/g TSS

(Bengtsson et al., 2018). The typical settling velocities are in the ranges of 12~145 m/h (Beun et al., 1999; Osman et al., 2001; Winkler et al., 2012). Because of the faster settling, AGS can form into higher biomass concentrations inside the reactor. Accordingly, it is feasible to operate the system with high organic loading rates (Maszenan et al., 2011). In addition, a wide range of SRT(sludge retention time) allows for a more diversified microbial community (Lourenço et al., 2015), including the slow-growth bacteria groups. This means less sludge is produced compared to CAS (Y. Q. Liu & Tay, 2007). From a microscopic perspective, AGS has dense and vigorous structure. This allows AGS to effectively handle industrial effluents and have a significant impact on toxic level (Beun et al., 2000; Y. Liu & Tay, 2004).

2.5 AGS

2.5.1 Granulation process

It was not completely understood of AGS granules formation process, but the four steps seen from below Fig. 3 show the general aerobic granule formation process. The main factors that contribute to aerobic and anaerobic granulation are EPS synthesis, quorum sensing, cell surface hydrophobicity, and ionic bridges (Sarma et al., 2017).

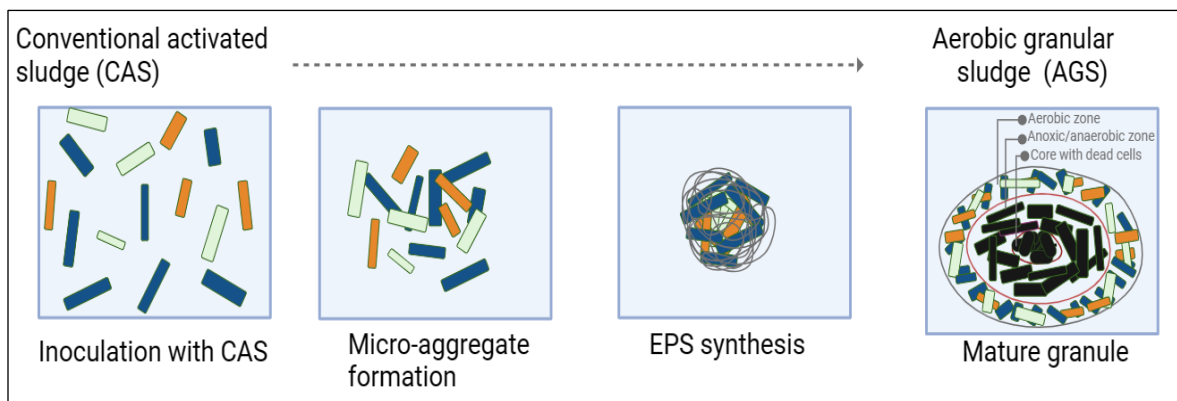


Fig.3 The Schematic representation of Aerobic Granule Formation.(Drawn by biorender.com)

The research on granulation process included organic loading rate, hydraulic retention time, selection pressure, and shear stress. The microbiological factors comprised the dominant microorganisms, EPS functionalities, and quorum sensing (Sarma et al., 2017). When certain physiological conditions are met, the microbial cells gather together and form multicellular groups (Calleja, 1984).

2.5.2 Startup strategies

It is well known that starting up AGS processes with real wastewater may consume quite a long period even by the means of inoculating active sludge (Bengtsson et al., 2018). Some studies showed that it took up to 6~13 months to accomplish 80% granular out of the whole biomass (Derlon et al., 2016; Y. Q. Liu et al., 2010; Ni et al., 2009; Rocktäschel et al., 2015; Wagner et al., 2015; Wagner & Ribeiro Da Costa, 2013). Comparatively, Beun et al. (2002) found that replacing substrates with synthetic wastewater allowed for only a few weeks to reach granulation. The factors affecting granulation duration are system temperature, selective pressure, organic loading rate, and varieties of inoculation taken during the granulation process (Bengtsson et al., 2018).

(i) High temperature

The mechanisms of the high-temperature boosting granulation process are due to the faster growth rates of the engaged microorganisms and the reduction of water's viscosity (Bengtsson et al., 2018).

(ii) High selective pressure

With high selective pressure, the flocs slow to settle down and removed. Eventually, the higher settling velocities microorganisms are selected. However, do not apply extremely short settling

time to avoid a large amount of biomass loss. Researchers proved the step-wise increase of selective pressure (by gradually reducing settling time) as an effective start-up strategy to achieve granules selection. It also prevents large-scale wash-out (Lochmatter & Holliger, 2014; Szabó et al., 2016).

(iii) High organic loading rate

Multiple researchers (De Kreuk & Van Loosdrecht, 2006; Y. Q. Liu & Tay, 2015) showed that high organic loading rates speed up the granulation. The organic loading rate has been gradually increased at AGS operations to meet the effluent standards. This method has been successfully in forming the majority of granules (De Kreuk & Van Loosdrecht, 2015; Giesen et al., 2013).

(iv) Granular biomass inoculation

With only a small number of granules inside the seed (5%~10%), granulations became essentially accelerated, and satisfying nitrogen removal performance was accomplished (De Kreuk & Van Loosdrecht, 2015; Giesen et al., 2013). The better that granules habituate to the wastewater, the shorter granulation process will occur (Bengtsson et al., 2018).

2.5.3 AGS stability factors

Up to now, the duration of healthy granules is strongly related to the wastewater characteristics. We need to fully understand how the granular disintegrations work, even though there are well-agreed mechanisms (Y. Q. Liu et al., 2005).

(i) C/N ratios and Organic loading rate

When the C/N ratios vary, the growth rates of autotrophic and heterotrophic microorganisms will

differ. Eventually, these differences will affect the granule's sludge biodiversity and microbial community distributions. In our study, the high C/N ratio meant a high organic loading rate, which made the heterotrophic microorganism dominant. Then nitrification or nitrification activities got suppressed, and possibly granules disintegrations occurred. As to the low C/N case, it resulted in insufficient denitrification or denitrification. Research done by Kocaturk and Erguder approved that, at the low COD/TAN condition, the TAN removal rate maintained at 100% for stable operations. Conversely, the high COD/TAN scenario reached only 33% TAN removal rate (Kocaturk & Erguder, 2016). Wei gradually increased the C/N ratios from 3 to 10, achieving desirable nitrogen and phosphorus removal rates accompanied by granulations of granules sludges (Wei et al., 2014). With a 3~13 kg COD m³ d⁻¹ loading rate, the system failed due to the granules disintegrations (del Río, 2013).

(ii) DO

Aerobic granule size decreases, filamentous bacteria outgrow, and density decreases under low DO conditions (40%). Granule disintegration also takes place. It eventually resulted in unsteady operations and biomass washout (Mosquera-Corral et al., 2005).

(iii) Granules size impact

When aerobic granule sizes grow larger than 1 mm, the stability can be degraded (H. Zhang et al., 2011). The filamentous microorganisms can bring adverse effects (Zheng et al., 2006). The reactor failed when the aerobic granules became 16 mm in size. The granules deteriorated and were washed out. The studies concluded that some contaminants inside wastewater can lead to granule disintegrations, for example, exposure to Fluoroquinolones (FQs) (Amorim et al., 2014). Schwarzenbeck et al (2005b) found that when AGS size increased dramatically, the reactor ended

with granule disintegrations.

(iv) pH

Exactly pH impact on the AGS stability is not fully understood yet. Optimized ammonia oxidation by nitrification takes place at a pH range from 7.5~8.0 (Metcalf & Eddy, 2014). When pH is higher than 9, the gel-forming EPS inside granules sludge gets hydrolyzed and leading to granule disintegrations. On the other side, when pH is in the 6.5~8 range, EPS exists as strong gel conditions, meaning granules in health situations (Seviour et al., 2009). The studies by the Lashkarizadeh group proved that, with the alkaline operation case (pH as 9), ammonia oxidation and COD removal rates all suppressed and were not recoverable. At a pH of 6, the nitrification rates and COD removal performance slightly declined, but not significantly (Lashkarizadeh et al., 2016).

(v) Temperature

De Kreuk 2005 investigated temperature impact on the AGS granulation plus short-term and long-term operation stability. The results showed that starting up at a higher temperature was beneficial to a more stable operation compared to the low-temperature case. The mechanism was believed that lower temperature resulted in the irregularly formed AGS associated with poor settling and easy washout from the system. The recommendations were to starting up the reactor or AGS process in warm seasons (summer preferably), which facilitates the faster metabolism activities of the microorganisms (De Kreuk, Pronk, et al., 2005).

(vi) Feast and famine mode

It believed that the SBR process allows a periodic starvation phase to take place (Tay et al., 2001). This starvation time is essential to aerobic granulation because of the fundamental impact on cell

hydrophobicity (Y. Liu et al., 2003). Based on the above theory, the feast and fast cycle was created to boost aerobic granulation. The intermittent feeding promoted the configurations of dense and compact granules (Mcswain et al., 2004). Researchers studied the step feeding strategy to treat wastewater. They also switched anoxic/oxic modes. Over 85% nitrogen removal for more than 70 days were achieved (F. Y. Chen et al., 2013). The mechanisms were due to the provision of carbon source for denitrification inside aerobic granule systems. These ideas were tested in lab and pilot projects (Lemaire, Marcelino, et al., 2008; Puig et al., 2004).

(vii) Other factors

a. The complexity of real wastewater

Lemaire R (2008) discovered that the structures of granules differed when treating abattoir wastewater compared to synthetic wastewater. Accordingly, the more diverse microorganisms of granules also varied from granules (Lemaire, Webb, et al., 2008).

b. Selective sludge discharge

To effectively manager AGS granules, It is suggested to release sludge selectively before they break apart (del Río, 2013). Various researches done related to above strategy. The hybrid methods kept the SBR system running smoothly for more than seven months with low-strength wastewater. The key strategy was to limit the AGS diameters smaller than 800 μm . The operation procedures were: first, discharge sludge from the top of the reactor during granules formation. During operation, remove the bigger, aging granules from the bottom part of the system. Last, assemble and select the younger, smaller granules retained inside the system (Zhu et al., 2013).

2.5.4 AGS research gaps

AGS technology has made big progress in past 20 years. They've worked on different projects in labs and on larger scale. More research is needed to prove AGS functionality because current studies lack real wastewater. The studies also vary in terms of organic loading rate, high strength ammonia wastewater treatment, temperature, and complexity of composition (Wilén et al., 2018). Furthermore, operation strategies need to be investigated in deeper insight to prolong granules stability. There are challenges with industrial wastewater because of the flocculated COD compositions. There is also a promotion of PAO/ GAO abundance. To address these issues, a plug-flow reactor with anaerobic/ aerobic modes can be used to select slow growth bacteria (Franca et al., 2018). Furthermore, researchers expect to learn more about the main polysaccharides in EPS that form granules. To ensure reliable operation, develop a process to detect early granule disintegration. This allows for corrective actions like reseedling with stored granules to minimize impact (Sarma et al., 2017).

Chapter 3 – Reactor Performance analysis and Microbial Related Measurements Methods Development

3.1 Introduction

To better understand what reactions are happening inside the reactor and the microbial community in activated sludge, we need to develop methods for analyzing the reactor's performance and extracting DNA. This chapter explains the leachate wastewater characteristics, reactor operation strategy, and materials & methods employed to evaluate the reactor performance, microbial activity, and DNA extraction process.

3.2 Materials and methods

3.2.1 Leachate sample collection and characteristics

Raw leachate wastewater was collected directly from the waste liquid flow through the base of a landfill in Alberta. The samples were transported and stored under 4°C before use.

The basic characteristics of the leachate are shown in table 1.

Table 1. The basic composition of landfill leachate

Parameter	Range
NH ₄ ⁺ -N (mg/L)	300-800
NO ₂ ⁻ -N (mg/L)	5-20
NO ₃ ⁻ -N (mg/L)	1-5
COD (mg/L)	3000-9000
Alkalinity (mg CaCO ₃ /L)	3500-8000
pH	7.3-7.8

It is noteworthy that seasonal variations in landfill leachate chemistry lead to flocculation in several aspects such as ammonia, nitrogen, COD, alkalinity, and color. The reactor was starting up at the end of July during our investigation, which was normal for the rainy season and indicated comparatively low COD and ammonia loading. The delayed start-up time was caused by these circumstances. Afterwards, the leachate generated increased loading to the AGS system during the dry season, when ammonia and COD loading were significantly higher.

3.2.2 Reactor configuration and operation

The setup of a column AGS reactor with a working volume of 4 Liters (Fig. 4) is shown as below. An air flow flowmeter was installed to tune the desired air flow rate, and the whole process was in timer control mode.

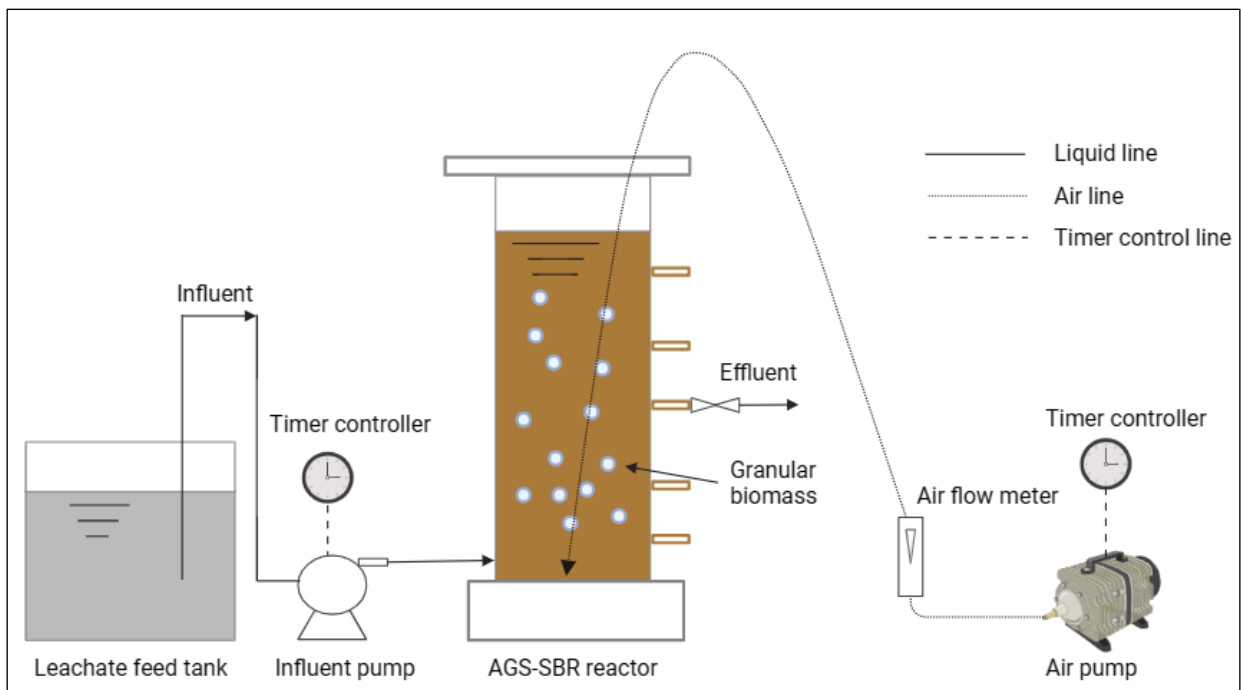


Fig.4 Schematic overview of column SBR nitrification reactor

The AGS reactor was inoculated with activated sludge from a full scale WWTP in Alberta and

anaerobic granules obtained from existing reactors during the startup phase. The exchange ratio was tuned at around 43% in sequencing batch mode (SBR) at influent 21°C. SBR mode is proven to be capable achieving reliable ammonia removal with simplicity, flexibility, less footprint and low energy consumption. HRT was 23 hours, and the solid retention time (SRT) was 20 days. After chemistry analysis, the alkalinity of leachate and pH were in the acceptable range. During the nitrification process, the airflow meter adjusted the amount of air distribution into the reactor. The reactor was in operation for over 200 days to evaluate the process feasibility, granules stability, and microbial community dynamics inside the reactor system.

3.2.3 Chemistry measurement

Raw effluent was collected to measure pH by utilizing a benchtop pH meter. Prior to the analysis, the influent water sample was taken from the feed tank; and the effluent water sample was gathered from the reactor effluent port; then all the samples taken were filtered through 0.45 µm filters. The chemical parameters such as $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$, and alkalinity were tested by Hach Kits (HACH company) and got reading values from a DR1900 benchtop spectrophotometer (DR1900, HACH, Germany). The procedure for $\text{NH}_4^+\text{-N}$ is 8038 and termed Nessler, while methods number 8153 (Ferrous sulfate), TNT 835, and TNT 870 were applied for $\text{NO}_2^-\text{-N}$, $\text{NO}_3^-\text{-N}$, and alkalinity analysis, separately. As to the COD detection, based on the Standard method 5220 D (APHA, 2017), the closed reflux colorimetric method was employed; utilizing the digestion solution mixed with $\text{K}_2\text{Cr}_2\text{O}_7$, H_2SO_4 , HgSO_4 , sulfuric acid and samples, after heating 2 hours under 150 °C, reading the abs values at 600 nm with DR1900 benchtop Spectrophotometer when the samples cooling down.

3.2.4 Solids measurement

Concerning the solid concentrations, the mixed liquor suspended solids (MLSS) was analyzed according to Standard method 2540 D (APHA/WEF/AWWA, 2018). The samples were filtered through a glass fiber and dried at 105 °C for overnight. Regarding the mixed liquor volatile suspended solids (MLVSS) analysis, the Standard method 2540 E (APHA/WEF/AWWA, 2018) was employed, igniting of 105 °C dried sample at 550 °C for 30 minutes to calculate the MLVSS concentration. As to the biomass measurements, firstly, to sonicate the biomass from the media; then apply the MLSS and MLVSS procedures to find out related results. The other variable of sludge volume index (SVI) was inspected based on the Standard method 2710 D (APHA, AWWA, 2004), monitoring the settled sludge volume within 30-minute settling period of 1000 mL mixed liquor.

3.2.5 Cycle test and activity test

To explore the reactions inside the reactor, in regard to the AOB activity test, the synthetic substrate consisted of 400 mg/L $\text{NH}_4^+\text{-N}$ (as ammonia chloride) and 1250 mg/L alkalinity as CaCO_3 (as sodium bicarbonate). The samples collected were filtered through 0.45 μm filters and analyzed for $\text{NH}_4^+\text{-N}$ concentrations. The ammonia oxidizing bacteria (AOB) activity was calculated using the $\text{NH}_4^+\text{-N}$ conversion rate divided by the biomass concentrations in flocs.

3.2.6 DNA extraction and microbial community analysis

At the end of stages with HRT of 14 h and 7 h, the genomic DNA of fresh suspended sludge was extracted using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Subsequently, DNA concentration and quality were assessed using the NanoDrop One (ThermoFisher, Waltham, MA), and the DNA samples were preserved at -20 °C until further

downstream analysis. The 16S rRNA genes from representative clones were sequenced at Genome Quebec (Montréal, Canada) utilizing the Illumina MiSeq platform with the universal primer-pair 515F/806R. Raw sequencing data underwent processing through the Qiime2 pipeline (Hall & Beiko, 2018) for read pairings after being filtered and denoised by the DADA2 algorithm (Callahan et al., 2016). Taxonomy was assigned using a 99% similarity threshold in the Greengenes2 reference database (McDonald et al., 2023). Alpha diversity analysis was carried out at the genus level using the ‘vegan’ package in R (Oksanen et al., 2007).

Chapter 4 – Results and discussions

4.1 Overall ammonia removal performance

The 4 L leachate reactor had a startup phase with below three stages, as seen in Fig. 5, targeting to remove the ammonia from the leachate samples.

Stage 1 was inoculated with anaerobic granules plus AGS sludge from an operating reactor in the lab. The ammonia concentration in the feed was 300 mg/L on average. The effluent ammonia concentrations became flocculated because of foaming issues. This eventually caused unstable performance.

Ammonia concentrations in the effluent were less than 50 mg/L for two months at stage 2, when only granular sludge were applied. Regretfully, the COD: N ratio was greater than 10 and the feed ammonia concentrations were increased to around 600 mg/L, which caused issues for the existing system.

During the final stage 3 of the startup phase, GSR sludge was selected and showed convincing ammonia removal rates within one week. It was proven that, with only 5 ~10 % granules, AGS start-up time period could be shortened dramatically (Coma et al., 2012; Pijuan et al., 2011). But the system failed as a result of the high C/N ratio. Next, the leachate was diluted with centrate to implement the approach of lowering the C/N ratio (obtaining the feed C/N ratio as round 2). The ammonia contents in the effluent were steady and less than 20 mg/L.

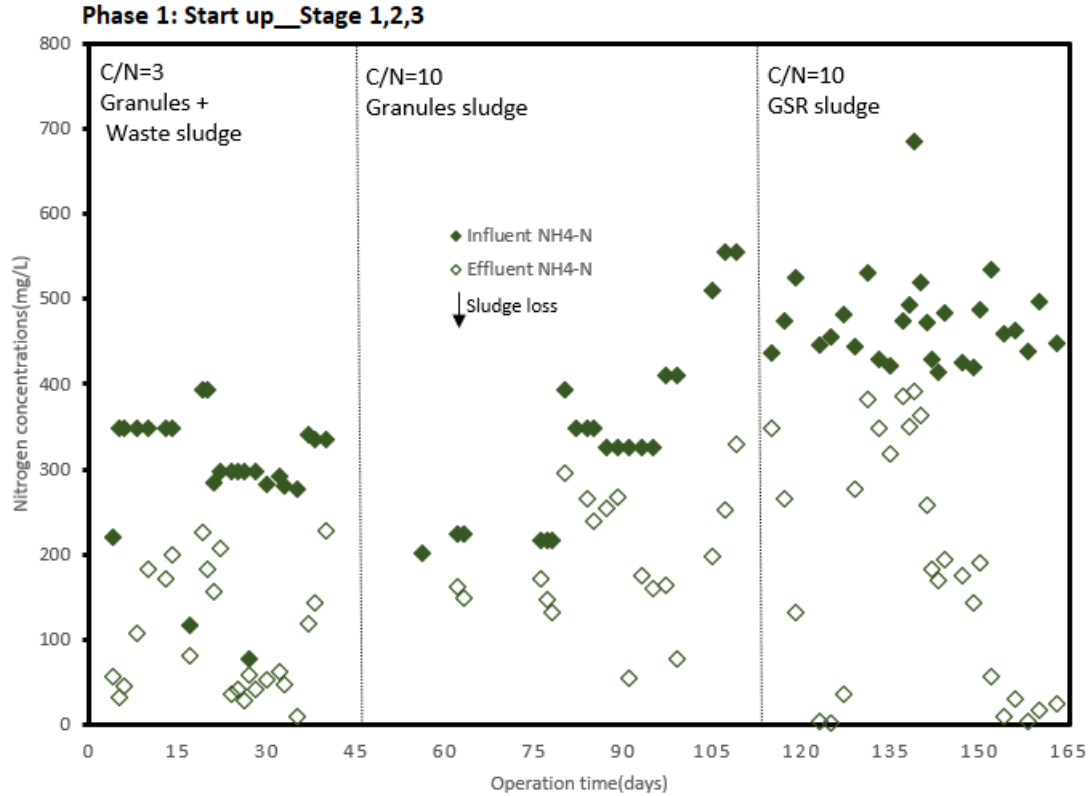


Fig.5 Reactor startup phase influent and effluent ammonia concentrations versus operation time.

Cycle tests were conducted throughout the operating phase to explore the possibility of lowering HRT without compromising the effectiveness of ammonia removal. The pH, ammonia, nitrite, and nitrate were monitored at predefined intervals throughout each cycle test. Careful analysis was then utilized to decide whether or not to shorten the HRT. By operating in this way, the system's HRT was effectively reduced from 23 hours to 14 hours, and then, after three months of steady operation, to 7 hours, resulting in a 98% rate of ammonia removal (Details show from below Fig. 6 a). Dong et al. (2018) recorded higher than 98% ammonia nitrogen removal rate using SBR-AO+ step feeding mode with the influent as 300 mg/L ammonia and COD/N 3 (AO meaning anoxic and oxic mode).

It should be noted that two sludge losses occurred. Because the selective pressure was maintained

constant during the operating phase, the system's sludge selection was the hypothesized reason for the sludge losses. Consequently, the entire biomass was unable to settle below the effluent port and be discharged with the effluent stream due to the combination of the new histotrophic biomass development, the existing biomass degradation, and the influent nbVSS buildup. It took around 10 to 15 days to recover the ammonia removal performance and back to stable operation again. With actual wastewaters, a definitive impact of selective sludge removal has not yet been documented. It is assumed that the type of primary solids removal and wastewater composition have an impact on the selective sludge removal's influence (Bengtsson et al., 2018).

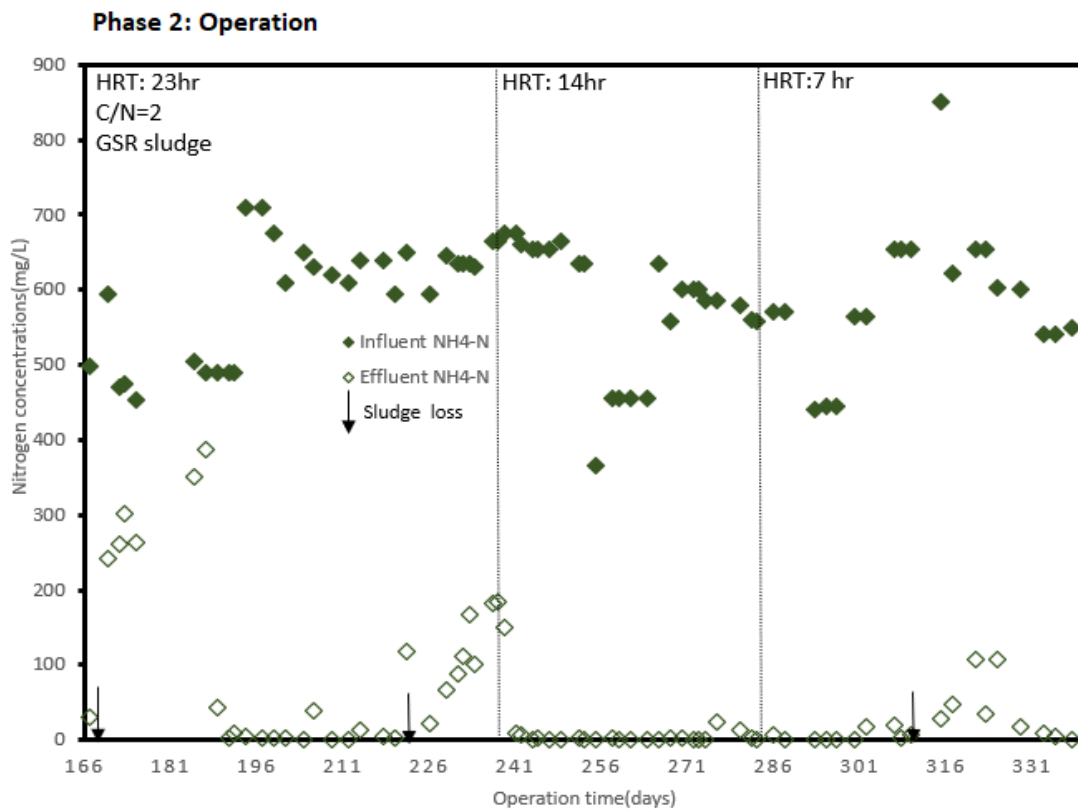


Fig.6 a) Reactor operation phase influent and effluent ammonia concentrations versus operation time.

The pH trend for influent and effluent during the operating phase was summarized in fig. 6b below. The average pH of the 83 samples collected over a period of 174 days was 7.7, with a maximum and minimum value of 8.7 and 6.2, respectively. Moreover, 42% of the pH of the effluent fell within 7.5-8, which is the ideal range for a dependable and stable nitrification process (Lashkarizadeh et al., 2016). No pH was more than 9, indicating that adjusting the pH had no detrimental effects on the disintegration of the granules (Dong et al., 2018b).

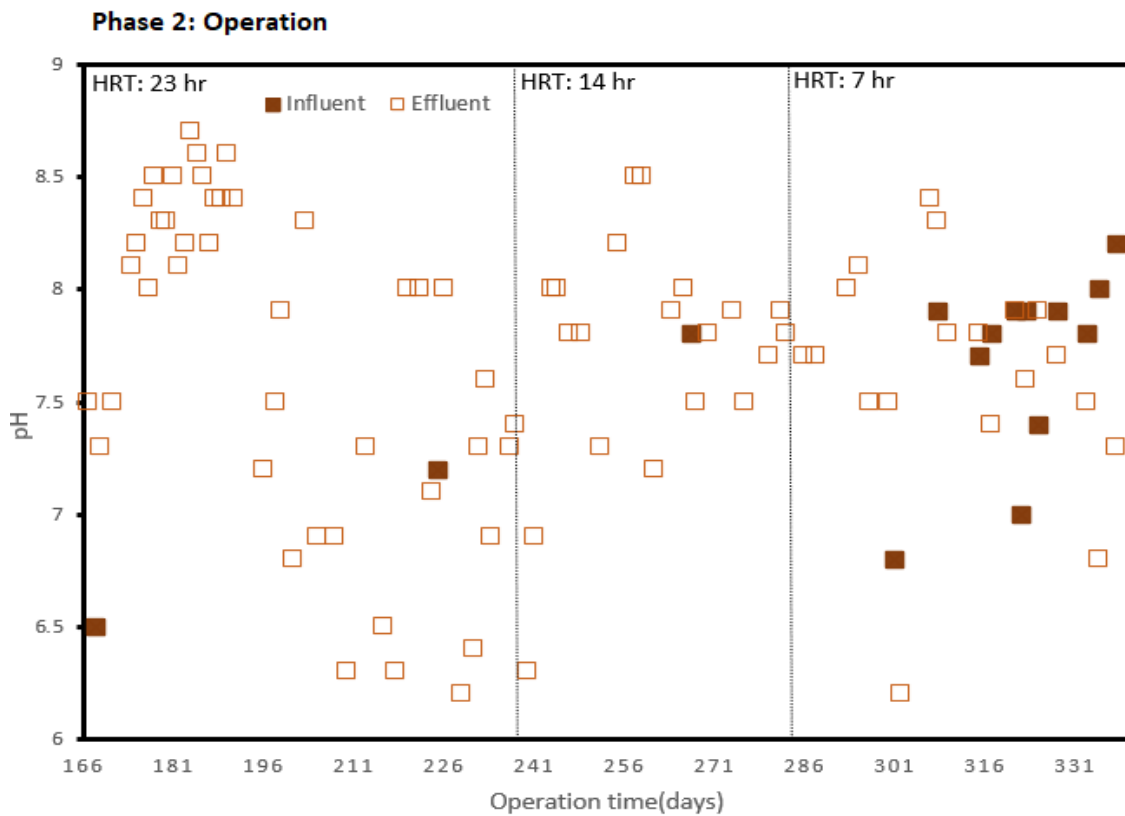


Fig. 6 b) Reactor operation phase influent and effluent pH versus operation time.

4.2 Cycle test study

4.2.1 Stable nitrification performance

At aerobic conditions, there could be many potential nitrogen mechanisms to occur, for instance,

nitritation, nitrification, denitritation, denitrification, comammox, anammox, and EPS hydrolysis. However, without the detection of related enzymes and functional genes (Song et al., 2021), this study is not capable of comprehending other processes, exclusively the stable nitritation at operation phase.

4.2.2 Sludge monitoring

SVI trend: It is well recognized that SVI is lower than 50 mL/g as the AGS distinction factor. Comparatively, the flocs sludge SVI values exceed 120 mL/g (Toh et al., 2003). As shown in Fig. 7 below, starting from days 166 after start-up, 75% of SVI₃₀ of AGS fell into this 50 mL/g range, indicating the granulations of AGS inside system were in good condition.

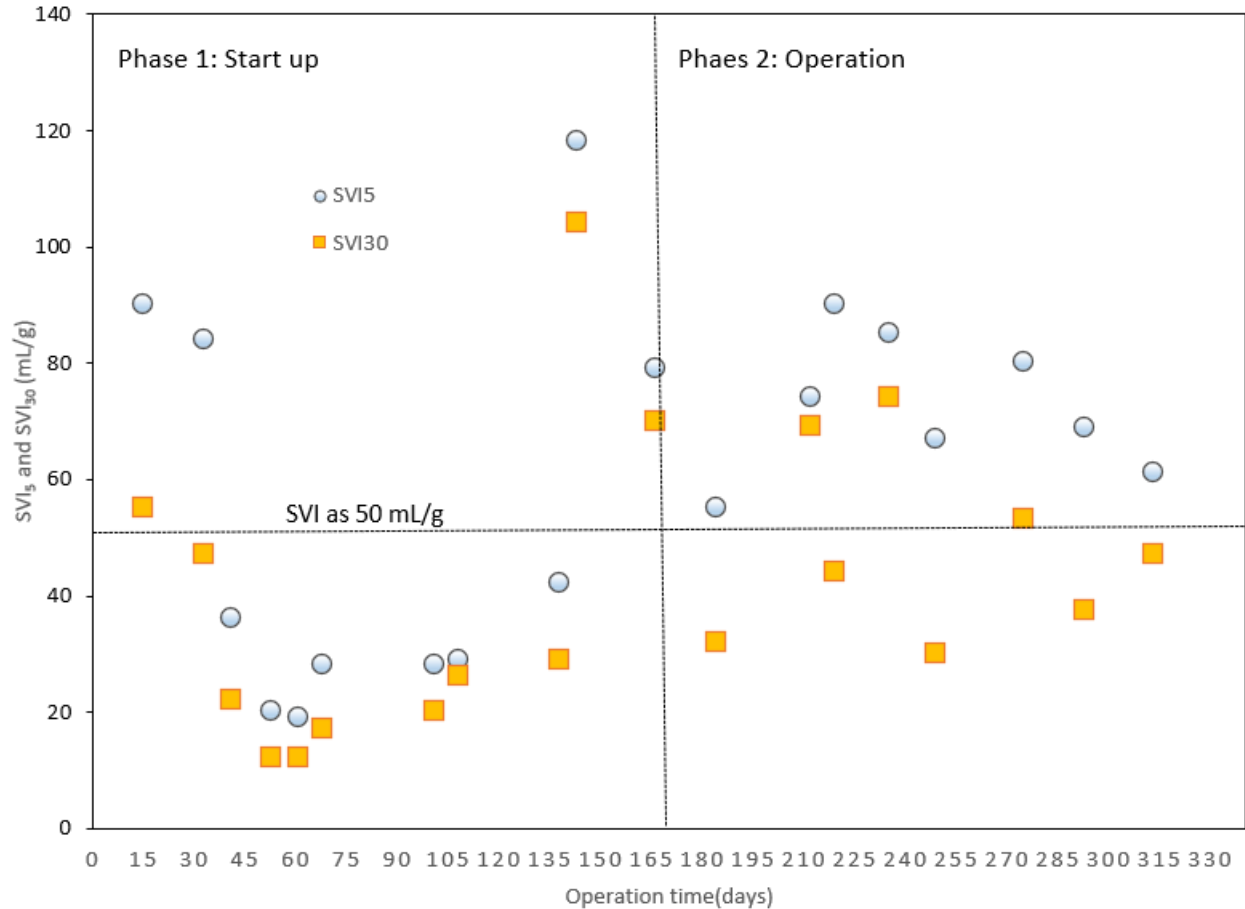


Fig.7 Aerobic granules sludge SVI trend along the operation.

Filamentous bacteria check: Examinations of filamentous bacteria under a microscope were conducted during the AGS system's beginning phase and were found to be nonexistent. When using actual dairy industry effluent, Schwarzenbeck et al. (2005) found that the filamentous granules formed at significantly lower OLR in comparison to Moy et al. (2002)'s results using synthetic water.

Viscous bulking: It was speculated that, at the startup phase stage 3, viscous bulking occurred and led to system instability. The vast extracellular biopolymer is what causes viscous bulking, resulting in a sludge that has a jelly-like, slimy consistency (Wanner, 1994). Water is retained in

the activated sludge due to the hydrophilic nature of the polymers. These sludges are poorly compacted, modest settling velocities, and a low density. Viscous bulking is often observed in systems that are nutrient-limited or in wastewater that has a high rbCOD content and a high F/M loading (Metcalf & Eddy, 2014) .

AGS size impact: The AGS sludge sizes in our reactor were identified small, which matched with Bengtsson et al. (2018) comments: granule diameters are often smaller after treating real wastewater with lower COD contents than treating synthetic substrates. The effectiveness of ammonia removal and granular stability, however, appeared unaffected by the lower size AGS.

4.3 Microbial activity

After batch tests, the highest nitrification activity was 27 mg N/g VSS/h at HRT in 7 h conditions. This value increased dramatically from 10 mg N/g VSS/h during the 23 h HRT operation phase. With AGS, complete nitrification has typically been easily accomplished. There is one study that is an outlier, in which the wintertime temperature dropped to 8 °C and the SRT was not high enough to sustain nitrification activity (Rocktäschel et al., 2015).

The relatively abundance of *Nitrosomonas* (2.1%) contributed to the high ammonia removal performance; also, the good settling ability (SVI as 45 mL/g SS) ensured the high sludge concentration of VSS as 12.5 g/L (S. Wang et al., 2017).

4.4 Microbial community analysis

Table 2 shows the different microbial alpha diversity indices in the sludge samples. These samples were from stages with HRTs of 14 hours and 7 hours. Microbial richness, a widely utilized measure

of biodiversity, quantifies the variety of species in a given sample simply based on species count (Fath, 2018). In the sludge sample with a 14 h HRT, we detected 126 genera, while the 7 h HRT sample revealed 116 genera. Two commonly employed richness indices for microbial communities are Chao1 and ACE, while the Shannon and Simpson indices reflect microbial community diversity. With the decrease in HRT from 14 h to 7 h, reductions in the Chao1, ACE, Shannon, and Simpson values were observed. The changes likely caused slow-growth microbes, like nitrite-oxidizing bacteria (NOB), to be washed out (Jubany et al., 2009). The Pielou index is a metric used to gauge the evenness of species distribution within a community (Yan et al., 2023). At an HRT of 7 h, the microbial community exhibited a lower Pielou index (0.45) in comparison to the 14 h HRT condition (0.47). This difference could be attributed to the enrichment of r-strategist microorganisms, which are rapid growers that respond swiftly to elevated substrate and nutrient levels (Bodor et al., 2020).

Table 2. Microbial diversity indexes in the reactor.

	Richness	Shannon	Simpson	Chao1	ACE	Pielou
14h	126	3.3	0.75	126	126	0.47
7h	116	3.1	0.73	116	116	0.45

Fig. 8 illustrates the phyla that exhibited a relative abundance greater than 1% in the sludge samples at HRTs of 14 h and 7 h. In both samples, the phyla displayed an identical order of dominance, consisting of *Proteobacteria*, *Bacteroidota*, *Deinococcota*, *Actinobacteriota*, *Chloroflexota*, and *Planctomycetota*. This observation aligns with findings from Liu et al. (2023) and Wang et al. (2021), which emphasize the significance of *Proteobacteria* and *Bacteroidota* (also known as

Bacteroidetes) as key components of AGS with primary dominance. Notably, *Proteobacteria* exhibited a remarkable dominance over other phyla, comprising 83.4% of the community at an HRT of 14 h and increasing to 86.5% when the HRT was reduced to 7 h. This suggests that *Proteobacteria* may possess greater resilience to environmental fluctuations and potentially toxic compounds. The predominance of *Proteobacteria* has also been reported in previous studies related to landfill leachate treatment (Wen et al., 2016; Zhang et al., 2013; Zou et al., 2022). Among the phyla depicted in Fig. 8, *Proteobacteria* emerged as the primary phylum responsible for nitrogen removal. *Deinococcota*, ranking third in abundance in both samples, played a pivotal role in the denitrification process (Qu et al., 2021). It is noteworthy that *Planctomycetota*, the phylum housing anammox bacteria, exhibited an abundance exceeding 1% among all phyla. However, further investigations are required to assess the activity of anammox in the reactor.

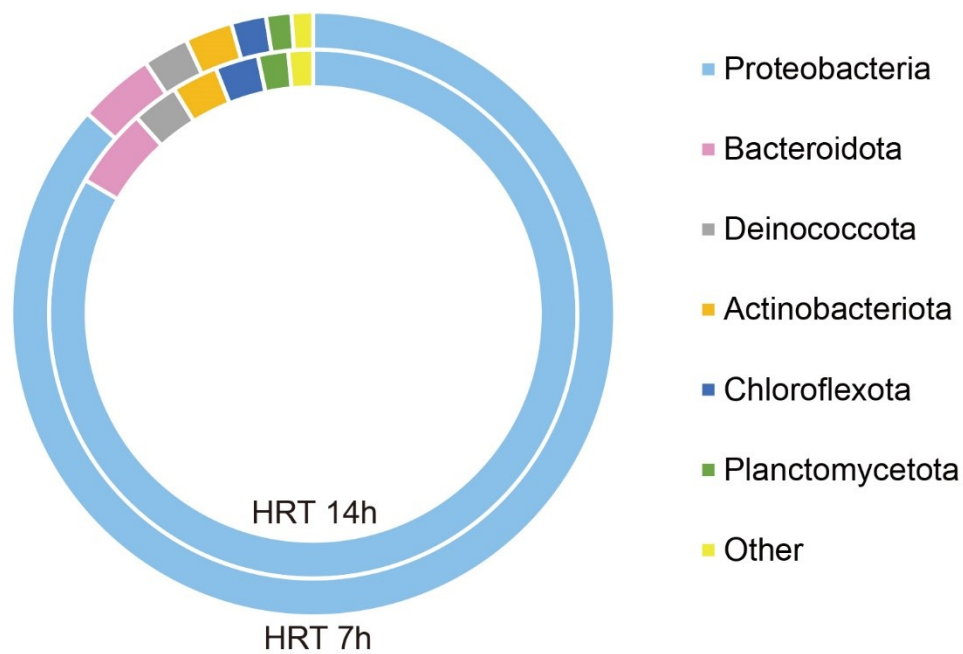


Fig. 8 Dominant phyla with abundance > 1% in both sludge samples at HRT of 14 h and 7 h.

Fig. 9 below illustrates the relative abundances (> 0.5%) of microorganisms at the genus level within the sludge samples. The genera *Thauera*_A_597130, a genus belonging to the *Pseudomonadaceae* family, and a genus from the *Burkholderiaceae*_A_592522 family were found to be dominant in both sludge samples. Remarkably, the genus *Thauera* exhibited overwhelming dominance among all detected genera at both HRTs, comprising 48.7% of the community at an HRT of 14 h and 39.5% at an HRT of 7 h. *Thauera*, commonly found in nitrification/denitrification reactors, has been noted for its role in achieving high rates of nitrogen and organics removal under anoxic or low dissolved oxygen (DO) conditions (Zhao et al., 2013; Zhou et al., 2018; Zou et al., 2022). Regarding ammonia-oxidizing bacteria (AOB) species, only *Nitrosomonas*, the most frequently found species in wastewater treatment processes (Lu et al., 2021; Pan et al., 2018), was detected in this study. The abundance of *Nitrosomonas* exhibited a slight decrease from 2.08% at an HRT of 14 h to 1.70% at an HRT of 7 h. This reduction in the relative abundance of *Thauera* and *Nitrosomonas* with decreasing HRT may be attributed to the washout of slow-growing biomass and their potential sensitivity to environmental changes or landfill leachate loading (Zou et al., 2022). However, it's worth noting that this decrease in their relative abundance did not impact ammonia removal performance. Interestingly, nearly all dominant genera exhibited decreased abundance with decreasing HRT, except for a genus from the *Pseudomonadaceae* family, which showed a significant increase from 16.1% at an HRT of 14 h to 30.5% at an HRT of 7 h. Denitrifying microbes within the *Pseudomonadaceae* family have been shown to be involved in denitrification processes (Adav et al., 2010). The higher relative abundance of *Pseudomonadaceae* family members indicated an increased potential for denitrification at an HRT of 7 h, consistent with the findings of Dong et al. (2017). Dong et al. (2017) reported that *Rhodocyclaceae* and *Pseudomonadaceae* were the dominant bacterial families in immobilized carriers and bioreactor

effluent, and as denitrifiers gained dominance, overall microbial diversity declined over time. Niu et al. (2018) also demonstrated that a reduced HRT condition favored denitrifier enrichment, leading to the rapid formation of denitrifying granular sludge. Therefore, the use of a 7 h HRT in this study may have been advantageous for granulation, thereby maintaining high ammonia removal efficiency.

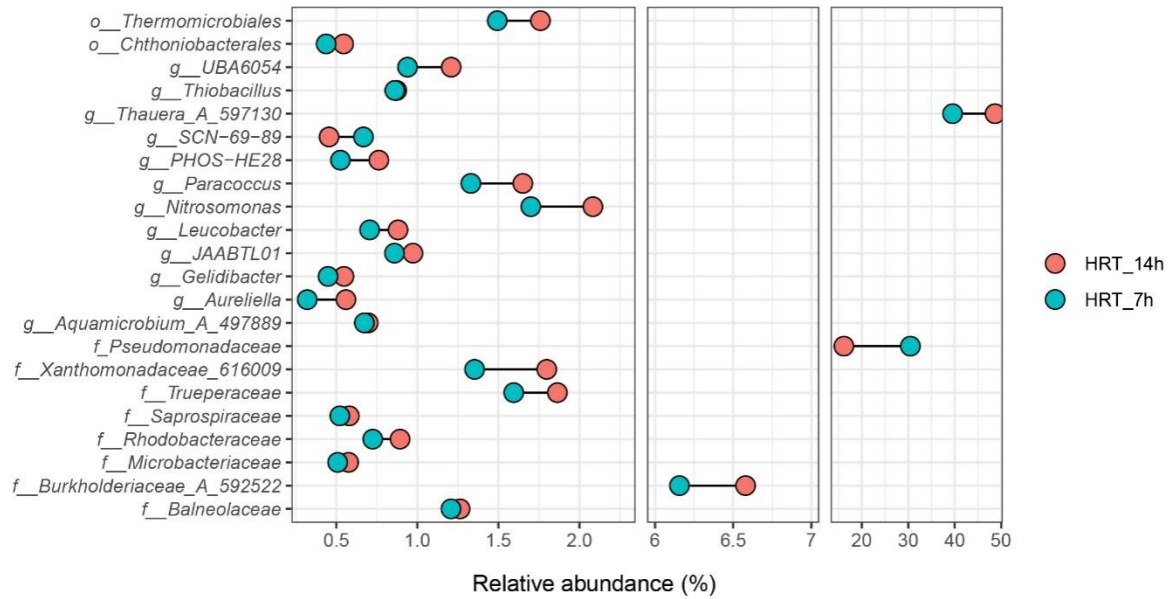


Fig. 9 Dominant genera with abundance > 0.5% in both sludge samples at HRT of 14 h and 7 h.

Chapter 5 – Conclusions and directions for future study

5.1 Conclusions

This study's primary goal was to assess the viability and stability of treating high-strength ammonia leachate with single-reactor nitrification. It will be extremely helpful to reduce the ammonia loading for the current WWTPs, which is brought on by the high strength ammonia leachate, with the quick and dependable ammonia removal employing SBR reactor. In this study the single reactor nitrification has been successfully illustrated in the AGS-SBR system reaching stable ammonia removal rate of 98% at very short HRT for more than five months operation time, without pretreatment of influent thanks to the resilience of AGS to deal with the complex and toxic leachate compounds. Furthermore, different start-up strategies were investigated; illustrating the importance of granule's inoculation, acclimation of AGS to the wastewater characteristics, and the quality of AGS making huge impact to the stable operation. The effective nitrification will occur when the system is high pH, high free ammonia (FA) concentrations, low dissolved oxygen (DO), and short SRT, eliminating NOB growth. Additionally, the microbial community analysis indicated a higher relative abundance of major AOB (*Nitrosomonas*) in suspended biomass, indicating the microbial distribution might be the core reason for the stable nitrification performance.

5.2 Future study

The study merely emphasized on the feasibility and stability of AGS single-reactor nitrification to treat ammonia-rich landfill leachate. Nonetheless, it is anticipated that a pilot size AGS nitrification test with online pH and DO monitoring will be done in the future, as pilot scale operation will offer more experience and data for possible full-scale operation in the future. Furthermore, the identification of functional genes should be established to better understand the exact reactions

occurring exclusively the nitrification process. Moreover, the hybrid methods of nitrogen removal could be explored such as nitrification and anammox in a single reactor, or AGS nitrification with membrane separation. Finally, as continuous AGS reactor is advantageous to full-scale AGS operation, additional research is needed on it.

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