

DEVELOPMENT OF HYDROGENOTROPHIC DENITRITATION PROCESS FOR MUNICIPAL WASTEWATER

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Abstract

With just about 2.5% of the earth's water, freshwater is the main source of water for all living organisms. Due to this fact, enhancement of wastewater quality is substantial towards maintaining fresh water quality and availability, since wastewater treatment plant (WWTP) effluent is discharged into surface water, hence disturbing the aquatic life, and plants.

Shortcut biological nitrogen removal has drawn research attention due to efficient removal of harmful nitrogenous compounds from wastewater by reducing energy and carbon requirements. In this thesis, the main objective is to develop a novel hydrogenotrophic denitrification system, in which high nitrite loading rate is reduced into nitrogen gas released to the air using a mixed bacterial consortium. This was proved first using a fed batch system followed by a sequential batch reactor (SBR) system under nitrite loading rate of $0.4 \text{ kg/m}^3\text{d}$. The nitrite removal efficiency reached up to 97% and a final nitrite concentration of $5 \text{ mgNO}_2\text{-N/L}$ and a specific denitrification rate of $45 \pm 4.5 \text{ mgNO}_2\text{-N/gVSS/d}$. The current study aims to achieve high rate nitrite removal using hydrogen gas. The proposed model is an important step to facilitate the coupling of this process with partial nitrification processes in a single reactor for side stream wastewater. This would lead to efficient nitrogen removal, and reduction in energy and aeration requirements.

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Acronyms

ANAMMOX	Anaerobic Ammonium-Oxidation
AOB	Ammonium-Oxidizing Bacteria
BNR	Biological Nitrogen Removal
CSTR	Continuous Stirred Tanks Reactor
F/M	Food to Mass ratio
HRT	Hydraulic Retention Time
NLR	Nitrogen Loading Rate
NOB	Nitrite-Oxidizing Bacteria
SBR	Sequential Batch Reactors
SHARON	Single reactor for High activity Ammonia Removal Over Nitrite
SRT	Solids Retention Time
WWTPs	Wastewater Treatment Plants
WAS/RAS	Waste/Recycled Activated Sludge

Chapter 1 Introduction

1.1. Background

1.1.1. Wastewater Treatment

Wastewater is an important water resource that needs sustainable management and improvement. Contamination of water resources happens due to number of reasons, which led to redirecting research focus and investments towards this area. Moreover, the growing concern towards climate change and global warming has driven global attention to wastewater treatment and development to help minimize the harmful effects on the environment.

Globally, wastewater can be collected, treated and finally used directly, or discharged to rivers and lakes where it integrates with the environmental flow and reused indirectly. One of the main constituents of wastewater systems is sludge which has a relatively high organic content as well as nutrients (nitrogen and phosphorous). This makes it an important asset for resource recovery plants from waste. Advanced treatment techniques enable wastewater recycling into secondary water uses, as well as resource recovery from nutrient rich wastewaters, these solutions offer economically feasible, and environmentally sustainable ways to the growing waste, and water scarcity problems (Alcamo et al., 2003; Mateo-Sagasta et al, 2015).

1.1.2. Nitrogen in Drinking Water

Nitrogen contamination of water resources has various side effects on humans, animals, and aquatic life, as well as air quality. Nitrite (NO_2), as a toxic compound, is a main cause of death to aquatic plants and fish, by causing oxygen inhibition to their cells and leading to diseases and ultimately death. Also for humans, it has a critical health risk as it causes a disease known as blue baby syndrome which could be fatal to infants, and is also associated to gastric cancer

(Karanasios et al, 2010; WHO, 2011). Moreover, excessive nitrite concentration from aquaculture and fish industries in lakes and water bodies lead to increase in oxygen depletion which causes eutrophication and excessive growth of algae which affects several aquatic communities (Visvanathan, Hung, & Jegatheesan, 2008; Zhou et al., 2011).

Also, nitrite accumulation beyond certain levels in groundwater and potable water leads to build up and release of nitrous oxide (N_2O) which is a greenhouse gas that contributes to ozone layer depletion and has adverse health and environmental impacts (De Beer et al, 1997). High concentrations of nitrogen highly limit the utilization of different water sources for drinking purposes. Therefore, there is a great need to remove these compounds from various types of water before disposing them into potable water sources (Ahn, 2006; Zhou et al., 2011).

1.2. Problem Statement

Conventional nitrogen removal processes impose numerous limitations, and hence they become less favorable alternatives for treatment. Physical-chemical removal technologies for nitrogenous compounds have high capital and operating costs, excessive energy demand, low performance in treating high organic or nitrogen concentrations. They also have operational problems such as large disposal volumes of brine waters, fouling and backwash, and pH variations (Bae et al , 2002; Hiscock, Lloyd, & Lerner, 1991; Shrimali & Singh, 2001). As for biological nitrogen removal (BNR), it is highly effective, inexpensive, and can be redirected into waste recycling and resource recovery by improving the design of the process. However, it has numerous problems such as slow growth rate for microorganisms leading to longer retention times, large reactor volumes required for treatment, high aeration costs, and production of organic residuals that need post treatment or handling (Ahn, 2006; Khin & Annachhatre, 2004). To cope with these drawbacks, various studies and research have designed bioreactors that enhance and

improve the efficiency of nitrogen removal with low costs and zero aeration and organic requirements.

Several reactors were able to improve nitrification, either by controlling aeration and reducing dissolved oxygen levels, or decoupling hydraulic retention time (HRT) from sludge retention time (SRT) using sequential batch reactor (SBR) setup, and controlling ammonia and nitrite loading rates so that the process is halted at nitrite accumulation stage. These approaches are known as partial nitrification reactors, such as ANAMMOX, SHARON, and CANON reactors (Ahn, 2006). The proposed reactor would be supplied with high ammonia loading rates, coming from side stream wastewater treatment, into a nitrite shunt reactor with controlled/low dissolved oxygen levels. After that, the reaction halted at high nitrite accumulation rates is injected with hydrogen gas (electron donor) in the presence of mixed culture bacteria, to reduce it into harmless nitrogen gas released into the air (Khin & Annachhatre, 2004; Sun et al., 2009). Denitrification related research are limited and focus solely on nitrate removal (i.e. electron acceptor) and heterotrophic cultures (i.e. organic) in aerobic denitrification reactors. The limitation on the aforementioned process is the high oxygen requirements, the organic by-products requiring post treatment, and the high operational costs from aeration and organic carbon source consumption. In this research, these problems are intended to be improved by reducing nitrite accumulated from shortcut nitrification reactors directly into nitrogen. This is achieved by microorganisms that utilize inorganic carbon source and hydrogen as electron donor.

1.3. Research Objectives

In this study, the main objective is to achieve a novel hydrogenotrophic denitrification process, mainly reducing nitrite from wastewater by using hydrogen gas as an electron donor. In order to do so, this objective had to be broken down to multiple milestones and minor objectives, as follows:

- 1- To eliminate the use of external organic carbon sources by using autotrophic electron donor which is hydrogen gas. This is due to the high costs associated with heterotrophic denitrification and the environmental impacts due to organic byproducts and intermediate gases produced.
- 2- To determine whether this process can be achieved with high nitrite loading rates (as in side stream wastewater treatment process) rather than conventional nitrate removal denitrification reactors.
- 3- To examine the different microbial strains that are able to utilize nitrite as electron acceptor in the presence of hydrogen gas and inorganic carbon source in the hydrogenotrophic denitrification process.
- 4- To identify the main factors to be controlled to increase the process efficiency in nitrite removal such as pH, alkalinity, and F/M ratio.
- 5- To develop and upscale the system in a SBR reactor. This could be integrated afterwards with partial nitrification process of side stream wastewater, to achieve a direct reduction of ammonia into nitrogen gas.
- 6- To study the feasibility of coupling hydrogenotrophic denitrification system with partial nitrification processes in a single reactor such as nitrite shunt reactors or SHARON process (single reactor system for high ammonia removal over nitrite process). This would lead to enhancements in energy and aeration requirements.

1.4. Thesis Layout

This thesis is composed of seven chapters in total. An introduction and research objective is presented in **Chapter 1**. After that, a comprehensive literature review on the topic of hydrogenotrophic denitrification and different approaches towards efficient nitrogen removal

processes in **Chapter 2**. In **Chapter 3** a study of hydrogenotrophic denitrification is developed in a batch setup, where parameters of the system are evaluated and monitored, and the effect of changing inoculated culture on nitrogen reduction rate is investigated.

Then **Chapter 4** describes the materials, methods and experimental setup of hydrogenotrophic denitrification, and the effect of changing food to mass ratio (F/M) on the performance and results of the experiment. **Chapter 5** presents another batch mode experiment where denitrification using hydrogen gas as electron donor is studied under different criteria. The effect of changing hydrogen dosage and influent nitrite concentrations is monitored, and the reduction rate of nitrite is analyzed in this experiment. Finally, **Chapter 6** depicts a study of hydrogenotrophic denitrification in a SBR system and entails its performance in nitrite removal using the investigated parameters.

In **Chapter 7** the main findings of the undertaken research are compiled and conclusion of the outcome with a glance into the future work, followed by the bibliography used in this research.

1.5. Thesis Contribution

This study provides an insight into hydrogenotrophic denitrification process, a novel approach for nitrite removal from wastewater using hydrogen gas and inorganic carbon source. The study aims to reaching high nitrite removal rates using SBR system. Up to date, this process has not been reported in the literature to reduce nitrite from water using hydrogen gas as an electron donor in a hydrogenotrophic environment. This is achieved by employing low concentrations of bicarbonate and hydrogen gas to remove high influent nitrite concentrations (up to 800 mgNO₂-N/L). Different electron acceptor (nitrite), donor (hydrogen) and carbon source (bicarbonate) concentrations were examined in order to identify their effect on performance of the process and

removal efficiency. The proposed process is an important step to facilitate the coupling of this process with partial nitrification processes in a single reactor and enables reducing ammonia over nitrite directly into nitrogen gas.

Chapter 2 Literature Review

2.1. Introduction

Nitrogenous compounds are found in the effluents of wastewater treatment plants, groundwater and sea water from various sources. It has become an increasing problem in many countries. This has driven the international community into setting stringent standards and limits for nitrates and nitrites in water to minimize and eliminate their effects. All of these combined have led to developing research to find better treatment and nitrogen removal processes. In order to do so, the background of these compounds has to be studied intensively to reach a deeper understanding of the process (Park & Yoo, 2009).

Naturally, nitrates can be found in plants, and in mammals' saliva. While artificially nitrate (NO_3^-) is one of the main components of inorganic fertilizers which find their way to surface and ground water streams through agriculture, human and animal wastes, and wastewater treatment. Explosives production and glass manufacturing are other examples of industries that utilize nitrate in the process. As for nitrite (NO_2^-), it is used in preservation of food in the form of sodium nitrite, especially in meat products where it is used to cure meat and inhibit growth of bacteria (Honikel, 2008).

Also, as a part of the conventionally occurring nitrogen cycle (displayed below) nitrate and nitrite are found. It occurs by nitrogen fixation naturally in air into ammonia which is utilized by most living organisms, or ammonia coming from organic human and animal wastes. Then this ammonia is oxidized into nitrite with the nitrifying bacteria, which is rapidly oxidized further into nitrate in a process called nitrification. The second part of this process is called denitrification where the nitrate is reduced in several stages into nitrogen gas. This process is

typically energy and resource consuming in terms of oxygen and carbon requirements.

Therefore, several research attempts have been developed over the years in order to facilitate this process in the most efficient, economic and sustainable ways possible (Karanasios et al, 2010).

While nitrite is an unstable compound, readily reactive, nitrate ion is the stable form of combined nitrogen for systems where oxygen is present such as fertilizer flow through groundwater and soil. Both compounds can be reduced into an oxygen free form by introducing microbial cultures or the proper physical & chemical reactions (WHO, 2011).

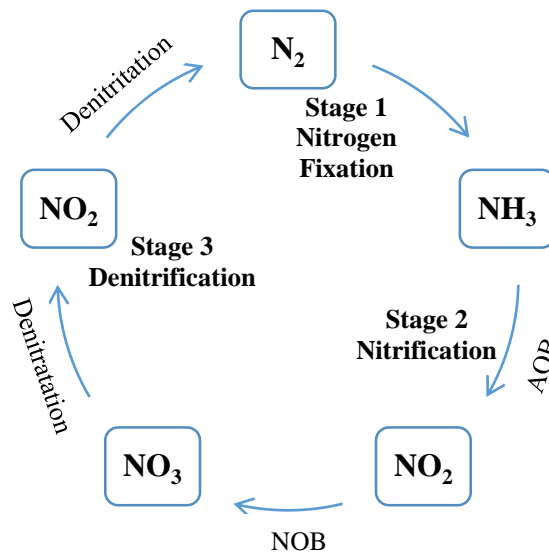


Figure 2-1 Schematic representation of the conventional nitrogen cycle

The dangers in the excessive nitrogenous compounds withdrawn in water streams is that they cause numerous problems for the aquatic system, as it leads to eutrophication causing excessive growth of algae and increase in the oxygen depletion and poisons in the aquatic life (Ahn, 2006). Moreover, they pose significant health risk to humans such as blue baby syndrome, and are suspected to cause gastric cancer; if present in excessive doses (Shrimali & Singh, 2001).

However, nitrogen removal from water is a difficult process, as they are present in form of ions dissolved in the water, not in solids that can be removed conventionally by filtration and settling. Therefore, removing nitrate (NO_3^-) and nitrite (NO_2^-) ions from drinking water has become a matter of high importance either by physio-chemical processes or biological ones. Due to its higher efficiency and lower cost over physical-chemical processes, Biological Nitrogen Removal (BNR) processes have been adopted widely.

2.2. Nitrate and Nitrite in Wastewater

2.2.1. Sources of Nitrogenous Compounds

Nitrogenous compounds can be present in water in variable forms, such as ammonia, nitrate and nitrite ions. In most water systems, nitrate is the final product of aerobic decomposition of organic matter containing nitrogen as per the nitrogen cycle. Nitrate (NO_3^-) is highly soluble, stable form of nitrogen and easily transported into groundwater or surface water through industrial, municipal discharge or landfill leachate. Groundwater contamination is linked mainly to agricultural activities as one of the main nitrogen sources in water is the use of fertilizers which is leaked to the groundwater beneath agricultural lands, hence the entire water cycle is contaminated. In soil, excess use of fertilizers containing inorganic nitrogen and animal or human wastes containing organic nitrogen are first decomposed to give ammonia, which is then oxidized to nitrite then nitrate. Excess nitrate then moves readily in the groundwater or surface water (USEPA, 1987).

Industrial activities are of the main contributors to nitrogenous compounds existence in water although they are not seen as important as agricultural activities. Since they are used in many industries as oxidizing agent especially nitrate such as plastic treatment, cleaning products, explosives and fertilizer manufacturing, metal finishing, and pharmaceuticals. These industries produce high strength nitrate. Some industrial wastewater contain nitrate in high ranges up to 1000 mg NO_3^- -N/L (Watanabe et al, 2001).

Contaminated land, such as old industrial sites or landfills can have a great contribution to groundwater contamination by nitrate and nitrite through leachate. Moreover, leakage from sewer networks and water supply systems has a high percentage of contributing into groundwater

contamination. As it is proven in many studies worldwide, leaky sewers provide the highest percentage of recharging aquifers. However, they leak polluted untreated water due to their deterioration, improper installation and/or high elevation above water table (Wakida & Lerner, 2005).

Nitrite is formed especially during disturbances, e.g., in nitrification bioreactors during oxygen depletion or ammonium overloading or suddenly increased levels of biodegradable organics; by denitrification during electron donor deficits; or in the presence of oxygen (De Beer et al, 1997). One of the ways nitrite can be formed is in water distribution pipes chemically by the *Nitrosomonas* bacteria that is found in organic wastewater, which is an autotrophic bacterium responsible for oxidizing ammonia into nitrite to maintain metabolism during stagnation of potable water and low oxygen in closed steel pipes. Also it has been reported that during tertiary treatment of wastewater i.e. disinfection, if chloramine is used as a disinfectant under low control or monitoring, nitrite could be formed in the presence of nitrifying bacteria with any residual ammonia in the system (Keinänen-Toivola et al., 2017).

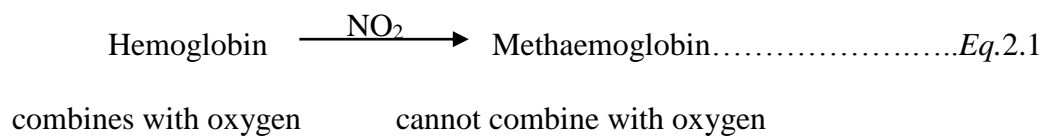
2.2.2. Harmful Effects

Nitrogenous compounds in water pose various risks to living organisms. Starting with nitrite (NO_2^-) in water, which is highly toxic for aquatic life such as fish, aquatic plants, bacterioplankton, and methanogens. The main toxic action of nitrite on aquatic animals, particularly on fish and crayfish, is due to the conversion of oxygen-carrying pigments to forms that are incapable of carrying oxygen, causing hypoxia and ultimately death. For fish, the entry of nitrite oxidizes blood hemoglobin into methaemoglobin which disables oxygen release to body cells. Similarly, in crayfish, entry of nitrite into the blood plasma leads to oxidation of

copper, whereby functional hemocyanin is converted into methemocyanin, causing a similar disease leading to fatality (Camargo & Alonso, 2006).

Nitrite can be present in high concentrations in sediments, also soil burrowing strongly increases efflux of nitrite from sediments, possibly by stimulation of nitrite formation by the increased variation in oxygen conditions induced by animal activity.

One of the critical health risks of nitrate is that it's converted into nitrite by bacterial activity inside human body in gastrointestinal tract, and in infants' stomach (Shrimali & Singh, 2001). This nitrite in the blood stream reacts with blood hemoglobin, inhibiting oxygen transport from the lungs to the body tissues as a result, which causes methemoglobinemia for infants (known as blue baby syndrome) (Burden, 1961). From the symptoms of this disease are, throwing up, facial muscles convulsions, irregular pulse, and difficult respiration. If the methaemoglobin in blood exceeds 5% of total blood constituents, these symptoms start occurring and if higher than 70%, it would cause death (Phillips, 1971). It is clearly seen that the main attribute to nitrate toxicity is its reduction to nitrite, which makes nitrite a highly toxic compound.



Also, nitrite in blood above certain limits might be associated with gastric cancer, but this risk needs further research and investigations to support these claims (WHO, 2011).

Moreover, some scientific evidences suggest that ingested nitrates and nitrites might result in mutagenicity, teratogenicity and birth defects, contribute to the risks of non-Hodgkin's lymphoma and coronary heart disease, and cause spontaneous abortions and respiratory tract infections (Camargo & Alonso, 2006).

One of the main environmental impacts is that high nitrite concentrations lead to buildup/accumulation of nitrous oxide (N_2O), which poses a global environmental risk, being a greenhouse gas involved in the ozone layer depletion (De Beer et al., 1997).

2.2.3. Limits and standards

Stringent limits and standards have been set worldwide not to be exceeded to control nitrate and nitrite existence in water sources, due to their aforementioned effects on living organisms and environment. Global organizations that set and monitor contamination levels such as World health organization (WHO), United States Environmental Protection Agency (USEPA) legislate these limits after doing thorough case studies, and research on the background levels and sources of exposure to these contaminants.

For nitrogenous compounds, these limits vary from drinking water to groundwater or wastewater due to the different routes and endpoint they are intended for. Health Canada (Health Canada, 2017) and US EPA adopt a similar limit for nitrate in drinking water; of 10 mg/L $\text{NO}_3\text{-N}$ (nitrate nitrogen), while (WHO) (WHO, 2011) and council of European communities (EU, 1998) has a limit of 50 mg/L nitrate (i.e. 11.3 mg/L NO_3^- -N).

As for nitrite acceptable levels in drinking water, the standard set by US EPA is 1 mg/L NO_2^- -N (nitrite nitrogen) (USEPA, 1987), while for the WHO is 3 mg/L as NO_2 (i.e. 0.91 mg/L NO_2^- -N). The most stringent limit for nitrite concentration is present in European Union which is 0.1 mg/L (i.e. 0.03 mg/L NO_2^- -N).

It can be seen that generally the exposure limits for US and Canada are stricter than the latter, which can be denoted to the gradual increase in nitrate levels in Europe over the last decades which almost doubled, in some countries the annual increase might exceed 1 mg/L in some water

bodies (EU, 1998). As for North America, the naturally occurring concentration of nitrogenous compounds do not go higher than 4-9 mg/L for nitrate and 0.3 mg/L for nitrite in general, which explains the variation in the limits seen above. In the United States, nitrates are present in most water sources below 4 mg/L except for some cases where it exceeded 20 mg/L in less than 5% of the surface or groundwater. But these cases are few therefore couldn't be relied on in deriving guidelines (Phillips, 1971).

Table 2-1 Summary of global limits of nitrogenous compounds

	US EPA/ Health Canada	WHO	EU
Nitrate (NO ₃)	10 mgNO₃-N/L	11.3 mgNO₃-N/L	11.3 mgNO₃-N/L
Nitrite (NO ₂)	1 mgNO₂-N/L	0.91 mgNO₂-N/L	0.03 mgNO₂-N/L

2.3. Nitrogen Oxides Removal Methods

2.3.1. Physiochemical Methods

Since nitrate and nitrites cannot be removed by conventional treatment processes such as filtration and sedimentation, more advanced techniques have to be employed. One of the treatment paths of nitrogen contamination in water is physical-chemical process. It can be done by several approaches such as ion exchange, reverse osmosis, electro dialysis, and catalytic denitrification. Nitrate removal by means of biological denitrification became commonly used for nitrate removal because it overcomes the limitation for physio-chemical process, as well as it produces nitrogen gas with a very high yield and low operational cost.

i) Ion exchange

In the ion exchange method, the water containing nitrate to be treated, is passed through bed of resins made of a strong base, then the nitrate ions replace the ions of the base (e.g. chloride or carbonate) until the resins' exchange capacity is reached by exchanging all ions (Kapoor & Viraraghavan, 1997). Although technically and economically effective, this method has two inevitable problems. The first is the trouble caused by sulfate ions where interfere heavily in nitrate removal. Typically, the ion selectivity for an IX resin is sulfate, nitrate, chloride, and then bicarbonate. Hence, treatment of high sulfate water with typical resins is difficult because the nitrate removal capacity of the resin is reduced by the sulfate ions. Therefore, the use of resins with nitrate selectivity over sulfate is preferred, which can be produced by increasing the number of carbon atoms around ammonium nitrogen in the resin structure, which can be a costly and time consuming process. However, several nitrate-to-sulfate selective (NSS) resins have been developed and applied to high sulfate waters.

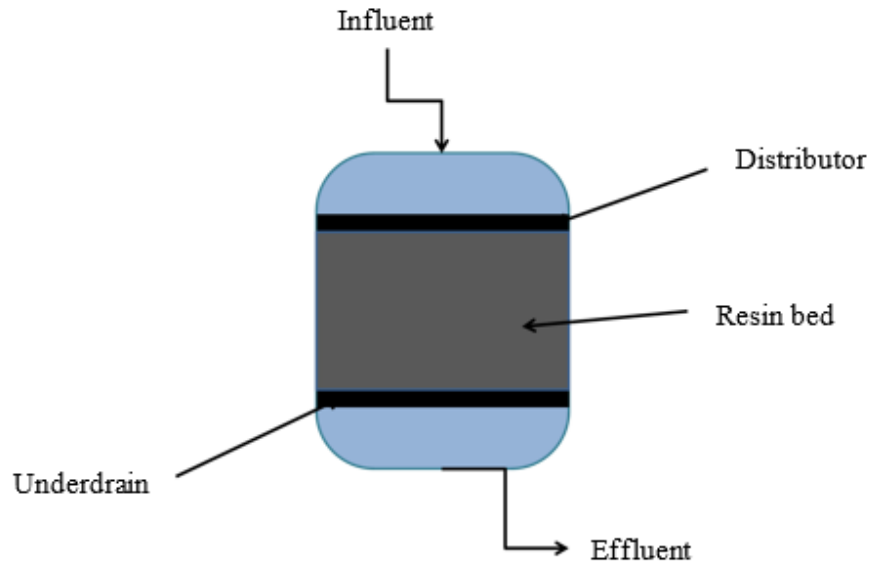


Figure 2-2 Schematic diagram of Ion Exchange Reactor

The second and most notable problem is the disposal of brine produced during regeneration of exhausted resins, which has high concentration of nitrates, sulfates and chlorides. To reduce the requirement for salt and brine disposal, several regeneration procedures and methods for recycling spent brine have been developed. To reduce the requirement for salt and later brine disposal, several regeneration methods for recycling spent brine have been developed. Van der Hoek extensively studied this problem and developed combined ion exchange and biological denitrification in an upflow sludge blanket reactor. It reduces the volume of brine produced by 95% (Van der Hoek et al., 1988).

The limitations in applying this method for denitrification can be summarized mainly into high capital and long term operational costs, excessive energy demand, pH variations, and large disposal volume of brine waters (Bae et al., 2002; Kapoor & Viraraghavan, 1997).

ii) Reverse Osmosis

As for the reverse osmosis which is wastewater treatment using impermeable or semipermeable membranes to remove solids, salts, large particles. Nitrate removal using this method is achieved by applying pressurized water to RO membranes exceeding the corresponding osmotic pressure. Its main limitations are its high capital costs, membrane fouling, pH variations and backwash leading to maintenance costs. Also, by time membranes deteriorate and remove desirable minerals and salts from water.

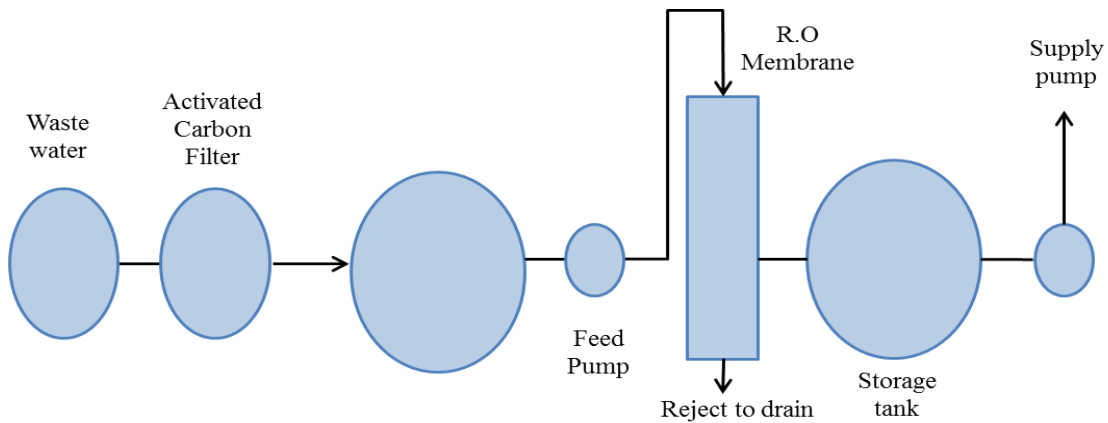


Figure 2-3 Typical Layout of Reverse Osmosis plant

These problems result from the deposition of soluble materials, solids, and other contaminants, and chlorine exposure; making the RO process not a desirable choice due to its high running costs (Kapoor & Viraraghavan, 1997; Schoeman & Steyn, 2003). The brine discharged from the process needs post treatment as for ion exchange method leading to extra costs, and it is inefficient in treating the low nitrate concentrations of wastewater (Park & Yoo, 2009).

iii) Catalytic denitrification

Developed in 1993, CD is a catalytic process for the removal of nitrite and nitrate from drinking water. Palladium- alumina catalysts were effective in reducing nitrite to nitrogen and ammonia in

the presence of hydrogen. The aluminum oxide catalyst was found to completely remove nitrate from water having an initial nitrate concentration of 100 mg NO₃/L (Kapoor & Viraraghavan, 1997).

In other studies redox catalysis has been used in the removal of nitrate from drinking water. These studies reported nitrate in water could be reduced to nitrogen gas in the presence of an appropriate catalyst. The use of a fine powder of Rh metal catalyst (5% on carbon black) was shown to selectively reduce nitrate (Peel et al, 2003).

The advantages of a catalytic reduction process are the rapid removal of nitrates from water without the production of waste byproducts. While the disadvantages of catalytic denitrification is mainly its ammonium production which would require further treatment leading to higher cost and energy use (Rocca et al, 2007).

iv) Electrodialysis

In ED ions are transferred through membranes from a less- concentrated to a concentrated solution due to the passage of a direct electric current. ED treats the water by selective removal of undesirable ions through a semipermeable membrane. An electrodialysis system requires a supply of pressurized water, a membrane stack and a direct current power source. The water requires pretreatment systems similar to reverse osmosis. A modification was introduced to the process into a reverse electrodialysis (EDR) process, where the polarity of the electrodes is reversed two to four times an hour to alter the direction of ion movement. The EDR process reduces scaling and chemical usage compared with conventional ED and has been used for the production of drinking water from brackish and seawater. The migration of ions is limited as anions can only pass the anion exchange membranes and cations only pass the cation exchange membranes.

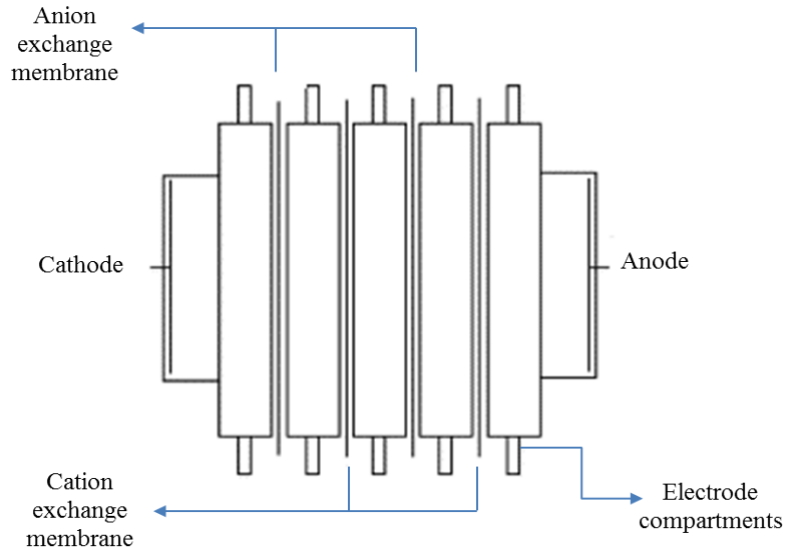


Figure 2-4 Layout of five compartment electro dialysis cell

The nitrate removal efficiency of ED and RO processes is more or less the same. The ED process is limited to treating soft waters, requires lesser acid dosages in comparison to RO and has higher water recovery rates. However, it is considered an expensive process, requiring close monitoring and long term operation and maintenance costs (Kapoor & Viraraghavan, 1997).

2.3.2. Biological Denitrification

Alternatively, biological methods have been adopted widely due to being energy and cost effective, and an environmentally friendly approach. **Conventionally**, denitrification occurs by facultative bacteria that utilizes nitrate (NO_3^-) as an electron acceptor for respiration using oxygen as an electron acceptor. Then, nitrate is reduced into nitrogen gas on 4 steps:

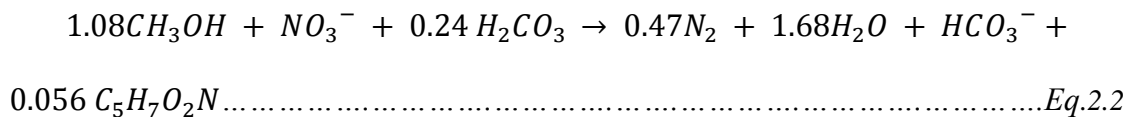
$\text{NO}_3^- \rightarrow \text{Nitrite } (\text{NO}_2^-) \rightarrow \text{Nitric oxide } (\text{NO}) \rightarrow \text{Nitrous Oxide } (\text{N}_2\text{O}) \rightarrow \text{Nitrogen } (\text{N}_2)$ (Karanasios, Vasiliadou, Pavlou, 2010).

The advantage that this process provides is that it treats nitrogen oxides, without production of toxic by-products, or the need of post-treatment. However, numerous experiment showed that biological process is a slower one when compared to physiochemical methods. This drove researchers to conduct extensive and thorough studies of the microorganisms governing the process, in order to select the most efficient ones, leading to higher and faster treatment rates.

i) Heterotrophic denitrification

One of the most common paths to achieving biological denitrification, is using heterotrophic bacteria. Heterotrophic denitrification utilizes nitrate or nitrite as terminal electron acceptors, and organic compounds as energy/carbon sources for bacterial growth, such as methanol, acetate and ethanol. Denitrifiers are common among the Gram-negative alpha and beta classes of the Proteobacteria, such as *Pseudomonas*, *Alcaligenes*, *Paracoccus*, and *Thiobacillus*. Some Gram-positive bacteria (such as *Bacillus*) (Ahn, 2006).

Methanol gained widespread use because it is considered cheaper than its alternatives. Typical stoichiometric equation for denitrification using methanol as an electron donor are as follows:



In (Eq. 2.2), the theoretical methanol requirement for nitrate is 2.47 mg CH₃OH per mg NO₃-N. In municipal wastewater and dairy-farming wastewater, where enough carbon sources for denitrification usually exist, control of the carbon to nitrogen (C/N) ratio is not a major concern for the process. Factors controlling the removal efficiency in this system are influent concentration, microbial concentration, the retention times of the sludge and wastewater, reactor configuration, etc. The influent to the treatment system in this kind of wastewater occasionally

also contains ammonia, which requires aerobic oxidation to nitrate and/or nitrite (nitrification) before it can be denitrified, making control of the oxygen concentrations another concern and a contributing factor to the operational and capital costs of this approach (Park & Yoo, 2009).

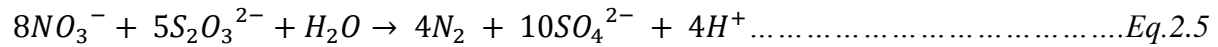
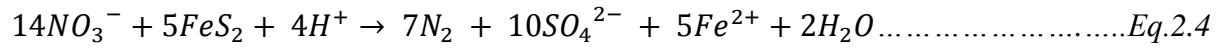
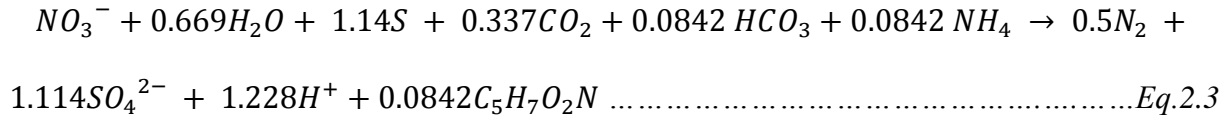
Its main advantages besides being commonly used, is being able to provide higher denitrifying rates and large volumes of treated water, as the denitrifying bacteria have high growth rates. But the by-products and residual carbon sources in treated water and the adverse effects they could have on humans, makes the use of heterotrophic microorganisms undesirable and potentially harmful. In addition to that, the organic carbon present naturally in the wastewater is quite limited, the complete removal of nitrogen from wastewaters that contain a high nitrogen concentration requires a large amount of an added organic carbon source for denitrification, which can be highly expensive compared to other biological methods (Kapoor & Viraraghavan, 1997; Khin & Annachatre, 2004).

ii) Autotrophic denitrification

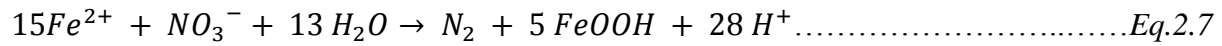
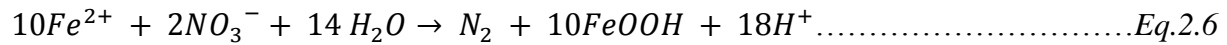
As a result autotrophic processes become more favorable, as they utilize inorganic carbon sources for their energy and electron requirements. This results in eliminating need for external carbon source, and lower handling costs for post treatment of sludge accompanied with heterotrophic method. Also, according to numerous studies lower sludge production is associated with autotrophic compared to heterotrophic per unit mole (Park & Yoo, 2009; Zhou et al, 2017).

Many compounds are used such as hydrogen, sulfur and iron compounds. Iron and sulfur have been used extensively throughout the years, due to their availability, high denitrification efficiency using inorganic carbon and energy source. For sulfur autotrophic denitrification, thiosulfate (S_2O_3), elemental sulfur and sulfide (FeS_2) can be utilized as electron donors, and

some of their stoichiometric equations (Eq. (2.3) – (2.7)) are given below (Ahn, 2006; Zhou et al., 2017).



And for iron as an electron donor:



Elemental sulfur was studied most extensively mainly because of its low price, high sulfur content to mass ratio among the reduced sulfur compounds, and the amount of sulfate produced in the process is least when elemental sulfur is used.

However, the sulfur approach has some limitations as it requires limestone as a buffer to increase pH during process, sulfur compounds have low solubility that limits its availability to microorganisms, and it produces sulfates which are harmful compounds for water, therefore further physical-chemical treatment needed, adding more cost and energy requirement to the process (Park & Yoo, 2009; Zhou et al., 2017).

While for the iron denitrification method, few research attempts have put it into trial. Its disadvantages are summed up into the production of ammonium which requires post treatment, really low pH affecting microbial activity and metabolism, and long duration needs for reaction to start (Kapoor & Viraraghavan, 1997; Park & Yoo, 2009). The aforementioned limitations could be avoided using the hydrogenotrophic denitrification process.

2.4. Hydrogenotrophic Denitrification

In this process, hydrogen gas is utilized as the sole electron donor for the bacteria, and nitrate or nitrite as terminal electron acceptor. It is a new alternative and process in comparison to its peers, but it is receiving high attention due to the pros offered by hydrogen gas.

2.4.1. Advantages and Limitations

There are several advantages to the utilization of hydrogen as an electron donor. On one hand, the low cost of hydrogen, in addition to its low yield of biomass, trigger the ease of applying hydrogenotrophic denitrification (Vasiliadou et al, 2006). Also, hydrogen gas poses no harm to humans or the environment, as well as no production of by-products or no requirement for removal of its excess substrate, which makes this process in favor of environmental sustainability. As for the economic aspect, it is less expensive than its counterparts, making hydrogen the most sustainable electron donor for treatment of drinking and wastewater (Ahn, 2006; Vasiliadou et al, 2006). For the application of wastewater, kinetic studies of the reactor design and the understanding of reaction mechanisms need to be gained in order to improve reactor efficiency.

On the other hand, some H₂ properties limit the application of hydrogenotrophic denitrification. Safety is an issue when it comes to hydrogen, due to its explosive nature if combined with air. In addition to that, it has poor solubility and diffusion in water (Park & Yoo, 2009).

2.4.2. Microbiology

Denitrifiers belong to a big versatile family of facultative anaerobic bacteria. They acquire their energy from electron transfer in redox reactions, to maintain and synthesize their cells. A variety of studies have been conducted characterizing the microbial nature in hydrogenotrophic

denitrification systems, where bacterial populations were isolated from mixed cultures used by this process. Most work has been done using mixed microbial cultures, whose population dynamics could change with the conditions. Results of these studies showed that most of the organisms with hydrogen-oxidizing denitrifying ability belong to a specific bacterial genera called Proteobacteria class. One of this class, *Paracoccus denitrificans*, is studied and used intensively as denitrifying microorganism. Populations of Proteobacteria, such as *Thauera sp.*, *Rhodocyclus*, and *Hydrogenophaga* were found in hydrogenotrophic reactors and were isolated to investigate their properties.

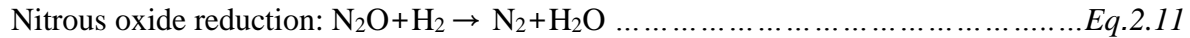
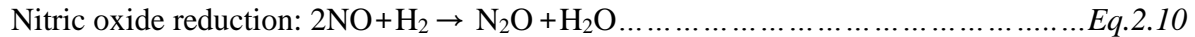
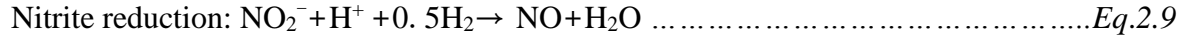
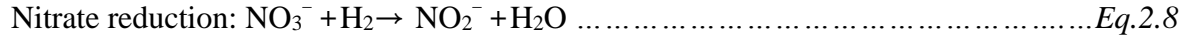
In multiple reactors, it was observed that *Paracoccus denitrificans* diminished in a hydrogen-dependent denitrification reactor where *Ochrobactrum anthropi*, *Pseudomonas stutzeri*, and *Paracoccus pantotrophus* increased and were thus in charge of denitrification. Also, some bacterial communities of the *Pseudomonas* genera, such as *Pseudomonas pseudoflava* and *Pseudomonas stutzeri* were observed in many reactors where hydrogen gas was used to treat nitrogen oxides, as well as *Alcaligenes eutrophus* which were mainly studied at first.

Nitrosomonas eutropha is an obligate lithoautotrophic nitrifying bacterium and also a denitrifying organism that uses hydrogen as the electron donor and nitrite as the electron acceptor (Ahn, 2006; Karanasios, Vasiliadou & Pavlou, 2010; Park & Yoo, 2009).

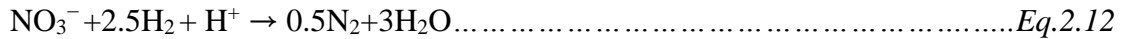
In conclusion, limited species of bacteria are able to perform hydrogenotrophic denitrification, due to its highly selective nature of a process. Those organisms should have capacity to utilize nitrate and nitrite as nitrogen source and terminal electron acceptor, while consuming inorganic carbon (carbon dioxide or bicarbonate) as their carbon source under anaerobic conditions, and also utilize hydrogen H₂ as their electron donor and source of energy.

2.4.3. Stoichiometry and Kinetics

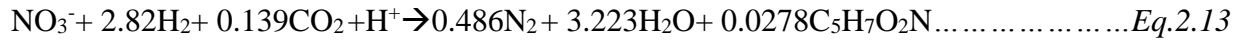
The basic stoichiometric reactions governing the processes are shown below: (Karanasios & Vasiliadou, 2010)



Making the overall reaction:



From Eq. (2.12), each mole of nitrate requires one hydrogen proton, typically 1 mg $\text{NO}_3\text{-N}$ would require 0.357 mg hydrogen. Also, from Eq. (2.8), H: N ratio for nitrate reduction is 0.14 mg H_2 /mg N, while for nitrite reduction 0.21 mg H_2 /mg N (Eq. 2.9). To further explain that in terms of pH and alkalinity for the nitrite reduction, pH would increase after the process as each mole of nitrate consumes one acid equivalent (H^+), to generate alkalinity of 3.57 gm as CaCO_3 per gm nitrate reduced. That's due to the fact that 1 mole of acid equivalent H^+ per 1 mole of nitrite converted to nitrogen. Also, alkalinity is released when nitrite is converted to NO, which can affect the system and cause precipitation or even clogging (Lee & Rittmann, 2003). For the bacterial metabolism, the autotrophic denitrification utilizes carbon dioxide or bicarbonate as carbon source; in the cell synthesis direction of the reaction. Several stoichiometric equations that have been developed in the literature, one of which is given below (Ghafari, Hasan, Aroua, 2009a).



In the above equation (Eq. 2.13), each g NO₃-N yields 0.22 g cells. Also, it illustrates that 2.82 mol H₂ and 0.14 mol CO₂ is required per mol NO₃, and the theoretical demand for hydrogen is 0.4 mg H₂/mg NO₃, and C:N ratio of 0.12. This shows that hydrogenotrophic denitrification can be achieved with low amounts of nutrients and carbon sources, however, more carbon source and electron donor are supplied to the system to prevent deficiency and ensure denitrifiers acclimatization. As a result, more experiment and research is still investigating this process to reach clear and solid stoichiometric values for the process.

2.5. Parameters affecting the process

2.5.1. Nitrate and Nitrite Concentration

The findings around the effect of the nitrate and nitrite concentrations vary as they have not been investigated thoroughly. Also, the kinetics of the process was not systematically investigated. In many studies, the initial nitrate concentration (NO₃) was the main factor under study and it had different results and effects until now. Being the terminal electron acceptor in conventional denitrification process, nitrate concentration continues to be studied to investigate its precise effect on performance of reactors and the potential that lies therein.

On the other hand, little to no research sought the nitrite concentration (NO₂) as a factor in designing the process, or its effect. However, its accumulation and final concentration during operation is monitored through some studies as reviewed later in this section. Some results show that high initial nitrate concentration did not prevent the reactor from performing and the bacteria were able to grow in these conditions. These trials changed the initial nitrate concentration between 20 and 492 mg NO₃⁻-N/L in order to track the reduction rate of nitrate. The results

showed that the nitrate removal increased with the increase of the initial concentration accumulating nitrite.

However, other results have shown that the high initial nitrate concentrations inhibit the denitrification process. It was found that full removal can be obtained at initial concentration of 10 mg NO_3^- -N/L. Concentrations more than 30-40 mg NO_3^- -N/L led to extreme nitrite accumulation and higher peak concentration.

2.5.2. pH

The denitrification process is influenced by the pH of the bioreactor. The most common ranges of the effective pH range between 7.6 and 8.6; however, since there are differences in the operating conditions and hydrogenotrophic cultures, the optimum pH tends to be around 7.5-8.5. This range prevents any inhibition to the reaction or nitrite buildup during the process. Higher pH, more than 8.6, can reduce the nitrate removal and lead to nitrite accumulation, while pH lower than 7 inhibits the reaction (Kapoor & Viraraghavan, 1997).

As a conclusion of the effect of the pH on the process, a controller should be used during the process to adjust the pH to the optimum value. Several controllers were tried to hold the pH as required. Phosphate buffers were tested in some experiments holding the pH in the range of 7 to 7.2. However, in some tests, low denitrification rates were observed as a result of mineral precipitation which affects biofilm density.

Other experiments used carbon dioxide gas as a buffer to avoid chemical usage in the process. The results of these tests showed better nitrate removal with no nitrite accumulation (pH stabilized in the range of 7) (Ghafari, Hasan, Aroua, 2009a). On the other hand, different tests concluded that carbon dioxide gas reduces the pH as low as 5.5 – 6 (Ghafari, Hasan, Aroua,

2010). Other trials to control pH introduced pyrite to the bioreactor to consume the hydrogen ions generated.

2.5.3. Alkalinity

Alkalinity is one the main factors affecting the denitrification process. As it shows the success of the process and whether denitrification is actually occurring and in favor of the bacteria or not. The amount of alkalinity needed per gm mole of nitrate or nitrite can be calculated from the corresponding stoichiometric equation for the utilized carbon source. Typically, 1 gm mole of nitrate consumes one acid equivalent (H^+), to generate alkalinity of 3.57 gm as $CaCO_3$ per gm nitrate reduced (Lee & Rittmann, 2003).

It causes a direct negative impact on the efficiency of the process if not controlled properly, because increasing alkalinity would increase pH of the system. This might lead to nitrite accumulation, rather than reduction, and would affect the bacterial metabolism and system stability. An experiment was done using hard water (with alkalinity range of 317.5 – 375 mg $CaCO_3/L$) and soft water (ranging from 145 – 165 mg $CaCO_3/L$). The hard water reactor did not proceed after few weeks due to the precipitation of $CaCO_3$ that caused clogging in the system. The increase of the alkalinity in the system may increase the pH which can affect the bacteria lifetime and produce chemicals in the waste that would need further treatment. This can be avoided by applying biomass synthesis or allowing $CaCO_3$ to precipitate and settle inside reactor (Karanasios, Vasiliadou, Pavlou, 2010; Lee & Rittmann, 2003).

2.5.4. Temperature

The experiments have shown that temperature affects the denitrification process since that it is one of the environmental conditions the bacteria are affected with. Normally, the denitrification

process takes place in the range 25 and 35 °C (mesophilic), but since the bacteria can survive severe conditions, denitrification process can occur in the range of 2 and 50 °C. Several trials were conducted to reach the optimum temperature for high nitrate reduction rate. Some results illustrated that the optimum denitrification rate was found at 42 °C, although it also occurred at temperatures as low as 10 °C (Kurt et al, 1987; Rezania et al, 2005).

Some other experiments demonstrated that the optimum range is between 30-35 °C, claiming that temperatures less than 30 °C could drive the reaction to the direction of high nitrite accumulation and temperatures more than 35 °C lead to lower nitrite reduction rates.

2.5.5. Hydrogen Concentration

The dissolved hydrogen concentration in the water affects the efficiency of the denitrification process. It is agreed that the minimum concentration of the dissolved hydrogen should not be lower than 0.2 mg/L. Concentrations lower than 0.2 mg/L results in incomplete process and increase of the nitrite concentrations. Some researchers have proved that the optimum hydrogen concentrations lie between 0.4 and 0.8 mg/L (Karanasios et al, 2011).

The effect of hydrogen supply varies according to the reactor design. In an experiment that used a membrane biofilm reactor, the low supply of hydrogen stopped the process as well as the growth of the biofilm.

On the other hand, the limited supply of hydrogen in a fiber membrane biofilm resulted in controlling the biofilm growth and increasing the performance of the reactor. In addition to the hydrogen concentration, hydrogen pressure is considered an important factor in the process. Better removal rates were obtained in the biofilm membrane reactor after increasing the hydrogen pressure from 0.45 to 0.56 atm (Karanasios et al., 2011).

An experiment was done in a submerged membrane bioreactor where the dissolved hydrogen concentration was between 0.2 and 0.55 mg/L. Complete denitrification occurred at lower concentrations, around 0.001 mg/L at the discharge. In other tests, it was detected that the biological activity causes an intense decrease in the hydrogen concentrations leading to nitrite acclimation (Rezania, Oleszkiewicz & Cicek, 2006).

A fluidized-bed reactor with an immobilized and isolated microbe, *A. eutrophus*, has also been tested and produced better rates but exhibited some limitations. Nitrate reductase and nitrite reductase suffered an inhibitory effect under 0.1 and 0.2 mg/L hydrogen, respectively, while hydrogen solubility in water is 1.6 mg/L. It was concluded that low hydrogen concentration caused nitrite accumulation and needed control (Park & Yoo, 2009).

2.5.6. Carbon Source

As mentioned earlier 2.4.3, the theoretical carbon demand for complete hydrogenotrophic denitrification is 0.20 mg C (in the form of bicarbonate) per mg nitrate. These mass ratios are low enough however higher ratios were used by researchers to ensure that carbon was sufficient for the culture to acclimate and process to happen.

Ghafari et al. studied the acclimation of autohydrogenotrophic denitrifying bacteria by using two inorganic carbon sources (CO₂ and bicarbonate) and hydrogen gas as electron donor. They observed that bicarbonate as the only carbon source showed a faster adaptation, while the use of carbon dioxide resulted in longer acclimation period. Usually, after the cultivation of microorganisms, the investigators try to find the optimum operating condition with regard to carbon supplies. They observed that bicarbonate is more appropriate for a faster growth and

adaption, however, a combination of bicarbonate and carbon dioxide has the ability to develop enough denitrification capacity (Ghafari, Hasan, Aroua, 2009a).

In another study conducted by same team, it was reported that the optimum bicarbonate concentration from a range 20 to 2000mg/L was 1100 mg NaHCO₃/L for an initial nitrate concentration of 20 mg NO₃-N/L (Ghafari, Hasan, Aroua, 2010).

However, experiments conducted by (Karanasios et al., 2011) showed that completed nitrate and nitrite removal was achieved with a mass ratio of only 0.504 mg C(as carbon dioxide)/mg NO₃-N, while dissolved carbon dioxide concentration ranged from 0.6 to 1.1 g/L.

2.6. Potential Bioreactors and Experiments

A number of bioreactors were recorded in the literature in study of hydrogenotrophic denitrification, specifically using attached growth systems due to their lower biomass yield. However, in this paper the focus is in regards to suspended growth systems due to their applicability in the proposed research, therefore both systems would be reviewed. Numerous adjustments and optimization of operating conditions and setups have been sought by researchers to achieve high performing hydrogenotrophic denitrifying reactor, and reduce any functional problems involved therein. Some of these trials, and research findings are analyzed in detail in the following section.

2.6.1. Suspended Growth Reactors

Suspended growth systems have been in application due to their availability and high organics/BOD removal up to 95% with the ability to treat nutrient rich wastewater with less operational problems. However, a limited number of research attempts and reactors were setup in

pursuit of measuring potential of hydrogenotrophic denitrification using suspended growth system.

Ghafari et al. studied the acclimation of autohydrogenotrophic denitrifying bacteria using inorganic carbon source (carbon dioxide and bicarbonate) and hydrogen gas as electron donor. In this regard, activated sludge was used as the seed source and sequencing batch reactor (SBR) technique to accomplish the acclimatization. Three distinct strategies in feeding of carbon sources were applied: continuous sparging of CO₂, bicarbonate plus continuous sparging of CO₂, and only bicarbonate. The pH-reducing nature of CO₂ showed an unfavorable impact on denitrification rate; however bicarbonate resulted in a buffered environment in the mixed liquor and provided a suitable mean to maintain the pH in the desirable range of 7–8.2. As a result, bicarbonate as the only carbon source showed a faster adaptation, while carbon dioxide as the only carbon source as well as a complementary carbon source added to bicarbonate resulted in longer acclimation period. Adapted hydrogenotrophic denitrifying bacteria, using bicarbonate and hydrogen gas in the aforementioned pH range, caused denitrification at a rate of 13.33 mg NO₃-N /g MLVSS/h for degrading 20 and 30 mg NO₃-N /L and 9.09 mg NO₃-N /g MLVSS/h for degrading 50 mg NO₃-N /L (Ghafari, Hasan, Aroua, 2009a).

The same team, in a trial to optimize the performance of the reactor, studied the nitrite reduction rate by optimizing pH and carbon source values in several experiments. This study successfully demonstrated that nitrite accumulation can be obviated in the denitrification process if the applied microorganisms were capable enough to perform a faster nitrite reduction than do nitrate reduction. In this regard, a 24 day acclimatization of a preconditioned mixed culture to nitrite doses of 5–20 mg NO₂-N/L confirmed this hypothesis and further investigation was focused on optimization of pH and bicarbonate dose in order to improve the nitrite reduction rate. Extremely

low reduction rates achieved at pH 6.5 and 8.5 entailed high susceptibility of denitrification towards pH and its driving influence on the reduction rate. Higher sodium bicarbonate doses showed better reduction rates implying positive effect of buffered environment on denitrification. However, it does not necessarily mean that the higher the dose, the greater the removal. Nitrite reduction rates obtained at pH range 7–8 were very close using 500, 100, and 1500 mg NaHCO_3/L . The highest specific rate was 25 mg $\text{NO}_2\text{-N}/\text{g MLVSS}/\text{h}$ achieved at pH 7.5 and 8 where 1000 mg NaHCO_3/L was used. A fairly close reduction time less than 4.5 h (> 22.22 mg $\text{NO}_2\text{-N}/\text{g MLVSS}/\text{h}$) was gained for the pH range between 7 and 8. After 11 sets of experiments, the highest specific nitrite reduction rate at 25 mg $\text{NO}_2\text{-N}/\text{g MLVSS}/\text{h}$ was achieved applying 1000 mg NaHCO_3/L at pH 7.5 and 8 (Ghafari, Hasan, Aroua, 2009b).

In their final study to optimize and study the kinetics of the system, they developed a model to predict the optimum conditions for the operation, and it predicted pH 8 and 1100 mg NaHCO_3/L . these values were compared to the experimental results and found that the process follows a zero order kinetic model with the ultimate specific degradation rates for nitrate and nitrite remediation were 29.60 mg $\text{NO}_3\text{-N}/\text{g MLVSS}/\text{L}$ and 34.85 mg $\text{NO}_3\text{-N}/\text{g MLVSS}/\text{L}$ respectively, when hydrogen was supplied every 0.5 h (Ghafari, Hasan, Aroua, 2010).

2.6.2. Attached Growth Systems

Operating conditions and apparatus information of several studies in autohydrogenotrophic denitrification using fixed-bed reactors are listed. The limitations associated with the use of fixed-bed attached growth systems are the difficulty in biofilm control, the limited mass transfer and the decreasing biomass activity due to thick biofilm formation. Experimental data showed that the use of the appropriate support media is of crucial importance for hydrogenotrophic denitrification, since it determines the extent of biofilm development as well as pore clogging. In addition the operating conditions (nitrate nitrogen concentration, volumetric flow rate) combined with a well-constructed configuration can enhance bioreactor performance (Mo, Oleszkiewicz et al, 2005).

In one study a fixed bed reactor was designed using glass beads to treat different nitrate concentrations in the range of 20–150 mg NO₃-N/L, the highest nitrate removal rate achieved was 0.225 kg N/ m³ d (H. Park, Choi, & Pak, 2005). In another study a cheap and effective installation using silicic gravel as support media was proposed, where the size of the support media was found to drastically affect denitrification efficiency. Using a triple-column reactor and multiple treatment stages shown below, high nitrate concentrations up to 340 mg NO₃-N/L were treated giving a denitrification rate of 6.2 kg N/ m³ d (Vasiliadou et al, 2009).

Most of these studies used the conventional hydrogen supply method which is an external tank for the gas direct sparging in the bioreactor. On the other hand, alternative methods for hydrogen diffusion have been proposed. Some reported studies used a tank where hydrogen gas is diffused via gas-permeable membrane and water would be hydrogenated. Afterwards, the hydrogenated water is used as an influent and fed to the fixed-bed reactor. An alternative mode was attempted in another fixed bed reactor, the hydrogen was produced in an electrolysis cell and subsequently

was introduced inside (Lu et al, 2009; Szekeres et al, 2001). One study self-produced hydrogen and carbon dioxide in their lab scale apparatus by the electrolysis of methanol, then these gases were sparged from the bottom of the reactor (Vagheei et al, 2010). Finally, the performance of a triple packed-bed reactor with hydrogen produced from electrolysis of water and electric power provided by a solar cell was investigated (Karanasios et al., 2011).

The use of inexpensive support media as well as the use of systems for cheap hydrogen production can make the hydrogenotrophic denitrification economically viable for potable water treatment. However, the limitations associated with the use of fixed-bed attached growth systems are the difficulty in biofilm control, the limited mass transfer and the decreasing biomass activity due to thick biofilm formation.

2.7. Conclusion

After conducting the literature study, identifying important parameters of hydrogenotrophic denitrification, some potential remarks and research gaps were noted. The study of nitrogen removal methods has been of attention throughout the years. Several methods of treatment have been applied in the past and results showed that biological denitrification is more beneficial, energy and cost efficient than physicochemical methods. Furthermore, trialed reactor technologies for denitrification were presented for both suspended and attached growth systems. However, up-to-date, the hydrogenotrophic denitrification process has not been developed to remove high concentration of nitrite using hydrogen gas. The current study aims to achieve high rate nitrite removal using autotrophic bacteria in a suspended growth system.

Chapter 3 Effect of using Different Cultures on Hydrogenotrophic Denitritation of Side Stream Municipal Wastewater

3.1. Introduction

Biological nitrogen removal has been applied extensively throughout the years due to its feasibility and advantages over other approaches. Hydrogenotrophic denitritation is a facultative anaerobic process where hydrogen gas acts an electron donor for microbial communities, which reduce toxic nitrite ions into nitrogen gas. This is the most economic and environmentally friendly approach and improvement on conventional denitrification, as it cuts down energy and aeration requirements, and utilizes clean and ecofriendly electron donor in the process.

Moreover, in this study autotrophic denitrifying bacteria, i.e. uses inorganic carbon sources (CO_2), is employed which is more efficient and better from environment and economic aspects than heterotrophic bacteria, i.e. organic carbon sources (methanol). However, the availability and optimization of the suitable carbon source and bacterial cultures is limiting factor in this process (Karanasios et al, 2010; Khin & Annachhatre, 2004; Lu et al, 2009).

Since hydrogen gas (the electron donor/reactant) and nitrogen gas (product of the process) are both clean and harmless gases to the environment and humans, hydrogenotrophic denitrification was highly studied and adopted in the recent years, and considered one of the environmentally friendly alternatives in comparison to other electron donors such as organic substrates and metals (Park & Yoo, 2009).

Previous studies have achieved shortcut nitrification and denitrification for nitrogen removal from wastewater. In a study conducted by Ghafari et al, autotrophic denitrification using

hydrogen was completely reached. It was conducted in a continuous SBR mode alternating carbon dioxide and bicarbonate in an attempt to identify the better carbon source at a rate 13.33 mgNO₃-N/gVSS/h for influent nitrate of 20-50 mg/L. The same research was later repeated the same study to improve the reduction rate and operation conditions, in this study nitrite removal at a rate of 25 mgNO₂/gVSS/h was achieved for influent nitrite of 20 mg/L (Ghafari, Hasan, Aroua, 2009b). In another study conducted by Vasiliadou et al, the effect of changing nitrate concentration and growth of mixed culture during which were investigated. In this research, the mixed culture used led to high efficiency in reduction rates when compared to other studies (Vasiliadou et al, 2009). It was concluded from previous researches that mixed culture has been more successful when it comes to inoculating denitrification reactors, as their diverse nature and dynamics proved high adaptation to the nitrogen concentrations and hard operating conditions such as pH and alkalinity control (Ghafari, Hasan, Aroua, 2010).

From research on hydrogenotrophic denitrification reactors and previous experiments, limited bacteria species were reported due to the highly selective nature of denitrifying environment, which requires the bacteria to be able to feed on nitrite as nitrogen source while acting as electron acceptor, and H₂ as electron donor. Also, these cultures should be able to utilize inorganic carbon source (such as bicarbonate) under anoxic or anaerobic conditions (Karanasios & Vasiliadou, 2010).

There's a lack of specific bacterial culture linked to this process in the literature to date. Consequently a study of different bacterial cultures' ability to denitrify nitrite into nitrogen gas using hydrogen gas as electron donor is crucial and going to be carried out through this paper. Five different bacterial cultures were selected to inoculate the batch fed reactors, two of which are of mixed nature, and three pure cultures as detailed in the following sections. A thorough

investigation and optimization of the parameters and proper organisms is a desired outcome of the experiment.

The effect of inoculum culture on the process of hydrogenotrophic denitrification will be investigated and illustrated, in order to identify the appropriate microorganisms.

For the selection of the microbial communities, the mixed culture coming from recycled activated sludge was the first type to be chosen, due to the common use of AS in hydrogenotrophic denitrification in many studies such as Karanasios et al, Park et al, Vasiliadou et al, and Ghafari et al. Therefore, identifying the strains and microbial culture associated became important (Ghafari et al, 2009a; Karanasios et al, 2011; Park, Choi & Pak, 2005; Vasiliadou, Pavlou & Vayenas, 2006).

The second type selected was ammonia oxidizing bacteria (AOB), that's due to the fact that hydrogenotrophic denitrification is a part of shortcut denitrification process that could be coupled with shortcut nitrogen removal reactors, such as nitrite shunt and SHARON reactors. These reactors' performance is highly dependent on the abundance of AOBs within, as it is responsible for oxidizing ammonia into nitrite in specific conditions. No previous study has researched the ability of AOB in reducing nitrite in the presence of hydrogen gas, therefore the selectivity of this culture was to be studied and confirmed (Ahn, 2006; Park & Yoo, 2009).

Methanotrophs have been isolated from denitrifying reactors that use methane and methanol as carbon source in aerobic conditions, but generally they have slow growth rate. Employing these types of microbial communities in hydrogenotrophic denitrification is under study, to study the potential of coupling methanotrophs and biohydrogen, produced from methane/biogas and anaerobic digestion reactors, to be used in reducing nitrite all in one reactor. This would be a novel economic and efficient partial denitrification approach (Khin & Annachhatre, 2004).

3.2. Materials and Methods

3.2.1. Experiment Setup

This experiment was designed in order to evaluate the ability different microbial cultures to use hydrogen gas as an electron donor, and nitrite as a terminal electron acceptor in a partial denitrification, namely hydrogenotrophic denitrification process. Enormous economic advantages would accrue if biohydrogen produced from different biological reactors could be used to remove nitrate from water, therefore attempts were made to isolate from various sources, bacteria which could utilize hydrogen in denitrification (Davies, 1973).

In this study, five types of biomass were used to investigate the potential of different bacterial cultures to reduce nitrite into oxygen gas using the biohydrogen as illustrated later in this section. The biomass used in all batches was acclimatized and preserved at 4°C cold room. The results and outcome of this study are presented in the following section.

The experiment was conducted in a batch mode in order to have a better control on the parameters, the operating conditions and the analysis.

The batch experiments were carried out in triplicates using 125 mL glass bottles capped with rubber septum to enable gas injection then covered with parafilm to enhance incubation.

These bottles were cleaned and autoclaved prior to each experiment to ensure the absence of any pathogenic activity. Afterwards, complete mix was controlled using Benchtop Orbital multi-shakers (Thermo Fisher Scientific, Waltham, MA) at 180 rpm, this speed was selected by conducting previous trials where this speed was optimum so as not to cause any separation or over mixing of the solution.

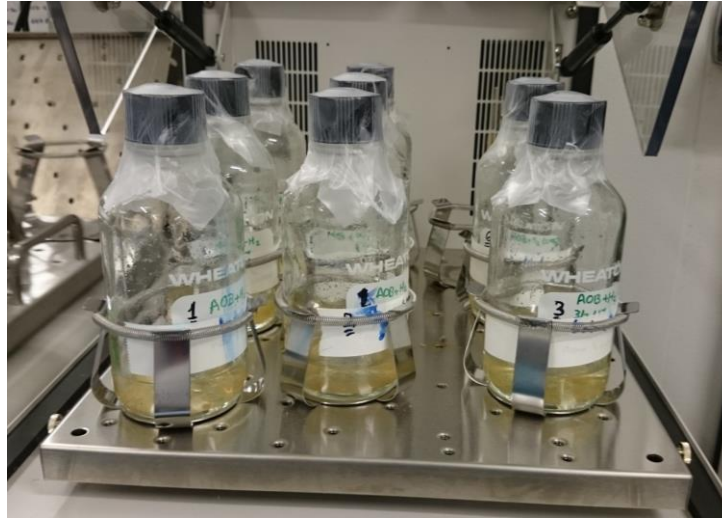


Figure 3-1 Triplicate batch samples placed on a multi-shaker

Due to the lack of previous studies in literature regarding cultures and strains relevant to hydrogenotrophic denitrification process, it has become important to investigate the cultures and microorganisms that are able to perform it. This stage consisted of five batches, each was conducted with one culture of biomass as shown in Table 3-1 which lists the influent parameters for each batch and biomass type.

Table 3-1 Experimental Setup and influent parameters

Experiment	#1	#2	#3	#4	#5
Biomass	RAS	AD	Type I	Type II	AOB
Nitrite (mgNO₂-N/L)	335±29	268±1.78	181±7.3	360±6.6	350±0
H₂ Dose (mL)	50				100
Alkalinity (mgCaCO₃/L)	2000				
F/M	0.4±0.04	0.4±0.09	0.4±0.06	0.4±0.08	0.15±0.05

3.2.2. Hydrogen Sparging

After adding the synthetic wastewater and biomass to each sample of 125 mL, the headspace was vacuumed from air and injected with chosen hydrogen dosage. 50 mL (40% of volume) was added to each sample in this set of experiments, using a gas tight syringe to reduce any leakage and impurities.

This volume was chosen after performing earlier trials and experiments, with different hydrogen dosages in which this value was able to maintain stable pH levels and microbial growth when employed with other parameters. Complete mixing of hydrogen was achieved by placing the samples after adding hydrogen gas on an orbital multi-shaker. This was done to ensure the diffusion of hydrogen into the solution, due to its low solubility and safety concerns.

3.2.3. Synthetic feed

The batch was inoculated synthetic wastewater with no organic substrate. The synthetic wastewater was freshly prepared by dissolving sodium nitrite, as nitrogen source and electron acceptor, into deionized water containing sodium bicarbonate as alkalinity source, potassium phosphate as phosphorus source, calcium chloride, and magnesium sulfate, as well as trace of mineral stock solution (Table 3-3) at a ratio of 1:0.001 per volume as shown in Table 3-2. The choice of bicarbonate as carbon source was based on literature studies which utilized carbon dioxide and bicarbonate, where sodium bicarbonate had better removal performance. This was justified by its nature as an alkalinity buffer for pH, which drops at the addition of hydrogen. As for carbon dioxide, pH variations would occur leading to the need of external buffer, and disturbance in microbial activity (Ghafari, Hasan, Aroua, 2009a).

Table 3-2 Synthetic wastewater composition

Compound	Concentration (per liter)	
NaNO₂	1971 mg	400 mgNO ₂ -N
	3942 mg	800 mgNO ₂ -N
NaHCO₃	850 mg	500 mg CaCO ₃
	1275 mg	750 mg CaCO ₃
	1700 mg	1000 mg CaCO ₃
	3400 mg	2000 mg CaCO ₃
KH₂PO₄	106 mg	
CaCl₂	53 mg	
MgSO₄	42 mg	
Trace elements	1 mL	

Table 3-3 Mineral Stock solution composition (for trace element)

Compound	Concentration (mg/L)
EDTA	15000
ZnSo₄.7H₂O	430
MnCl₂.4H₂O	990
FeSo₄.7H₂O	500
CuSo₄.5H₂O	250
CoCl₂.6H₂O	240
Na₂MoO₄.2H₂O	220
NiCl₂.6H₂O	190

Na₂SeO₄	210
H₃BO₄.7H₂O	14

3.2.4. Biomass and Seed

Five different seeds coming from different sources were utilized to inoculate the batches, according to the scope of each experiment. In this phase, the driving concept is studying the potential of pure and mixed culture to achieve hydrogenotrophic denitritation and comparing their performance.

The 1st biomass was a waste/recycled activated sludge (RAS) which was thickened to a high solids content of 10 gVSS/L. The second biomass employed was mixed culture coming from anaerobic digestion (AD) process. Both RAS and AD sludge were freshly collected from Humber wastewater treatment plant, Ontario, Canada.

Pure cultures were selected as inoculum for remaining experiments. Methanotrophs type I and II were cultured and used for the 3rd and 4th experiment, and lastly culture collected from a nitrite shunt reactor halted at nitrite accumulation stage, rich with ammonia oxidizing bacteria (AOB). For biomass density measurements, the methanotrophs have low solids content, for that reason identifying their TSS and VSS was not attainable. Optical densities (OD₆₀₀) was measured instead for types I and II. A correlation between optical densities and solids content is hence obtained.

All biomass samples were preserved in a cold room at 4°C before running the experiment. The table below (Table 3-4) entails the total suspended solids (TSS) and volatile suspended solids (VSS). Also, the respective hydrogen to microorganism (H/M), nitrite to microorganism (N/M) ratios for each of the cultures are calculated using the solids concentration.

Table 3-4 VSS and Optical densities for bacterial cultures

Type	OD₆₀₀	VSS (g/L)	N/M	H/M
RAS		9.2	0.04	0.004
AD		3.8	0.11	0.009
AOB		7.3	0.05	0.005
Type I Methanotrophs	2.74	1.3	0.31	0.03
Type II Methanotrophs	2.61	1.25	0.32	0.03

3.2.5. Microbial Community Analysis

One sample was taken from the enrichment stage of each mixed culture used to perform microbial sequencing. It was taken on the fourth day when the nitrite removal rate was the highest in comparison to previous days, to ensure the dominance of denitrifying organisms in the mixed culture.

The DNA extraction and the amplification of the V4 region of the 16S SSU rRNA were carried out using the Earth Microbiome Project benchmarked protocols. The DNA extraction protocol involved mechanical and enzymatic analysis followed by a phenol-chloroform extraction and a cleanup step using a MoBio PowerMag soil DNA isolation kit as per the manufacturer's protocol (Stearns et al., 2015).

Primers 515FB and 806RB were used, and the barcodes incorporated into the forward primer enabled the usage of various reverse primer constructs to obtain longer amplicons and removal of biases (Walters et al., 2016). Briefly, each 25- μ L PCR mixture contained the following to

amplify V4 of the 16S rRNA gene by PCR: 13 μ L of PCR-grade water (Sigma, St. Louis, MO), 10 μ L of Platinum Hot Start PCR Master Mix (2 \times) (ThermoFisher, Waltham, MA), 1 μ L of Template DNA, 0.5 μ L of 515FB primer (10 μ M), and 0.5 μ L of 806RB primer (10 μ M). The reaction then was run for 35 cycles (94 $^{\circ}$ C for 2 min, 94 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 30s, 72 $^{\circ}$ C for 30 s), with a final polymerization step at 72 $^{\circ}$ C for 10 min. The products were separated by electrophoresis in a 2% agarose gel and visualized under a UV transilluminator light. The products corresponding to the amplified V4 (300–350 bp) were excised and purified using standard gel extraction kits (Qiagen, Netherland). The products were then quantified with the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher). Equal amounts of product from each sample (240 ng) were combined into a single, sterile tube and then cleaned using MoBio UltraClean PCR cleanup kit. The final concentration was measured using a nanodrop spectrophotometer, and the product quality was checked by ensuring that the ratio of absorbances at 260/280 nm ranged from 1.8 to 2.

The resulting PCR products were sequenced using the Illumina MiSeq personal sequencer (Illumina Incorporated, San Diego CA) at the McMaster Genomics Facility, Ontario, Canada. Cutadapt method was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100 bp (Martin, 2011). Sequence variants were then resolved from the trimmed raw reads using DADA2, an accurate sample inference pipeline from 16S amplicon data (Callahan et al., 2016). The DNA sequence reads were filtered and trimmed based on the quality of the reads, error rates were learned, and sequence variants were determined by DADA2. Chimeras were removed, and taxonomy was assigned using the RDP classifier against the SILVA database version 128.

3.2.6. Analytical methods

Samples were taken from influent (before starting experiment) and effluent stage (on a day to day basis), and collected in airtight bottles daily, and refrigerated at 4°C until analysis was completed.

Total suspended solids (TSS), and volatile suspended solids (VSS) were analyzed according to the Standard Methods (APHA, 1998).

For Types I and II methanotrophs, optical density was measured to find out their cell densities, using HACH DR 3900 Benchtop Spectrophotometer at wavelength of 600 nm (OD₆₀₀).

As for gaseous measurements, samples were withdrawn with gas tight syringe from bottles headspace, then injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, CA) equipped with thermal conductivity detector (TCD) (Restek, Bellefonte, PA.) to measure hydrogen and nitrogen concentrations. The device was setup to the following temperature program: injector: 80°C; Oven: 80°C; FID: 300°C; TCD: 155°C. Helium and nitrogen were used as carrier gas with flow rate of 20 mL/min for nitrogen and hydrogen measurements, respectively.

As for alkalinity, it was measured using titration method with 0.01 N H₂SO₄ solution in accordance with the Standard Method no. 2320 (APHA, 1998). Alkalinity and pH were measured instantly in all samples using an installed (Mettler-Toledo, US) and pH-11 series pH/(mV · °C) meter (HACH HQ440d, US), respectively. HACH methods and testing kits (HACH DR 3900 Benchtop Spectrophotometer) were used to measure nitrite ion concentration (NO₂-N), nitrate ion (NO₃-N).

Dilution was done for the analysis in order to be within the range of the apparatus and methods used. For example, nitrite was diluted by 1:100 ratio, while COD was diluted for 1:2, and nitrate for 1:10 ratio. All batch experiments were carried out in triplicate sets, to satisfy reproducibility and ensure results' precision.

Each time-point in a single profile is also the average of triplicate sample measurements. Three profiles were made for each batch representing nitrite, alkalinity and pH. The error bars in the graphs denote 95% confidence of the average values.

3.3. Results and discussion

3.3.1. Batch Performance

For this set of experiments, it was run by inoculating the batch with a number of different cultures. For each experiment, the performance, and potential of the used culture to reduce nitrite was monitored and measured. Mainly, all parameters such as alkalinity, nitrite were kept constant to be able to have consistency in the performance of different inoculum and better control over the experiment.

The experiment started by setting up a batch for one complete run with the same HRT and SRT of 4 days allowing bacterial activity for nitrite reduction. The batch were inoculated with loading rate of 100 mg/Ld. The overall performance was in favor of mixed culture, specifically waste activated sludge (RAS), as explained in the following sections.

3.3.2. Nitrite Removal Rate

In the conducted experiments, nitrite reduction was observed with the highest efficiency up to $88\% \pm 2$ for experiment 1 inoculated with recycled activated sludge (RAS). It didn't exceed 50% for the other four cultures, with Type I methanotrophs being the highest as seen in Figure 3-2.

Keeping alkalinity and nitrite concentration constant, it is observed that when influent nitrite concentration was set to 400 mgNO₂-N/L, RAS adapted with a fast removal rate over 4 days and removed around 90% of the nitrite concentration.

Final nitrite concentration reached 45 ± 5 mgNO₂-N/L for RAS mixed culture, which is considered the lowest, and 200 ± 13 mgNO₂-N/L for AD mixed culture counting for ~25% removal efficiency. As for pure cultures, Type I methanotrophs final nitrite concentration reached 90 ± 3 mgNO₂-N/L leading to ~50% removal, while almost no removal (<10% nitrite removal efficiency) was observed in Type II batch samples. As for culture collected from ammonia oxidizing process (AOB), the removal efficiency was around 30% reaching final concentration of 238 ± 6 mgNO₂-N/L.

This supports results of other experiments conducted with activated sludge culture presented in other chapters, where complete nitrite removal was achieved under similar conditions, making the RAS culture more suitable for operating conditions of auto-hydrogenotrophic denitrification. Further analysis for other parameters governing process is done.

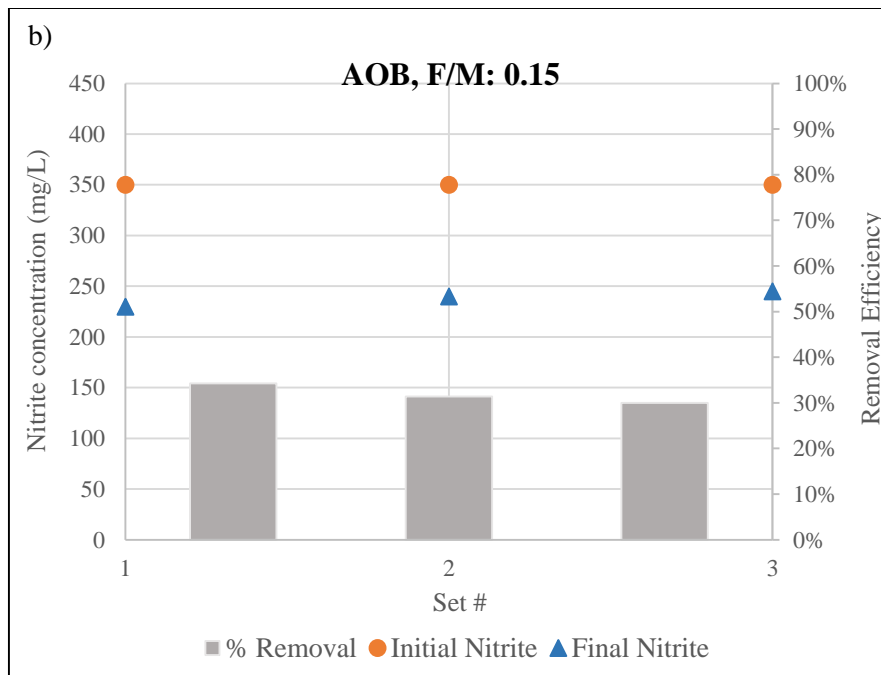
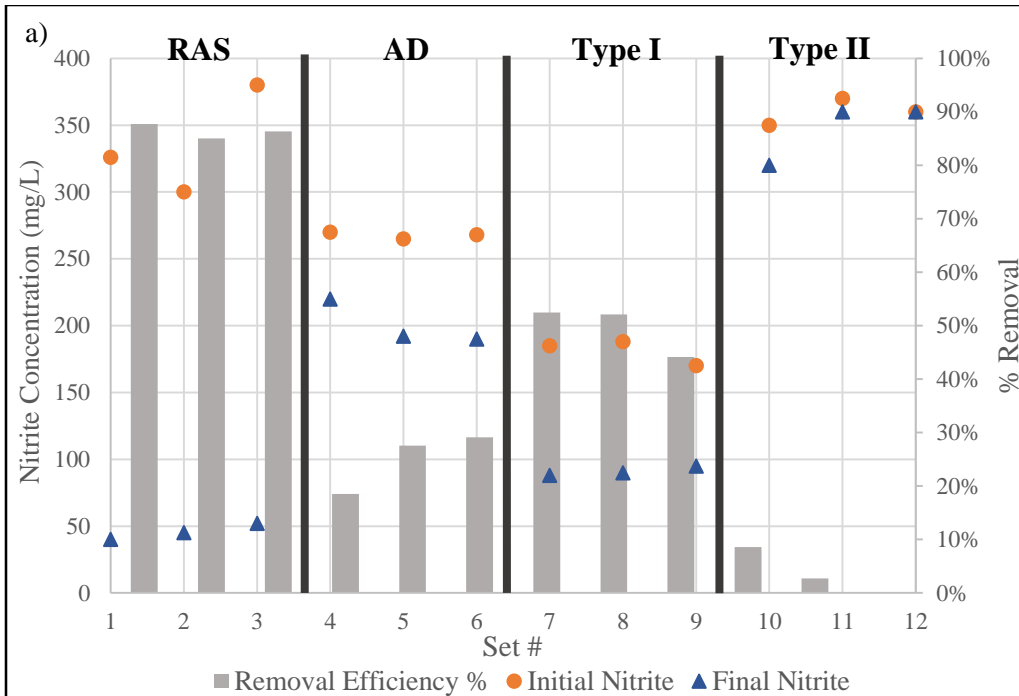


Figure 3-2 Nitrite removal profile for: a) the first 4 cultures of F/M: 0.4 gN/gVSS (RAS, AD, Type I and Type II), b) AOB pure culture of different food to mass ratio (F/M: 0.15 gN/gVSS)

3.3.3. Effect of Inoculum Cultures on pH and Alkalinity

Alkalinity and pH were monitored as they are of the main parameters to measure the performance of hydrogenotrophic denitrification and bacterial activity. According to (Karanasios & Vasiliadou, 2010) the optimum pH level for this process is within the range of 7.5-7.6, and higher pH above 8.6 might lead to nitrite accumulation and inhibition of nitrite reduction.

As for alkalinity, typically 1 gm mole of nitrate consumes one acid equivalent (H^+), to generate alkalinity of 3.57 gm as $CaCO_3$ per gm nitrate reduced (Lee & Rittmann, 2003). Since denitrification is in favor of producing alkalinity in terms of $mgCaCO_3/L$, therefore a measure of the potential of these cultures to achieve proper denitrification is an increase in alkalinity concentration of the wastewater. However, an excessive alkalinity production might cause operational problems such as minerals precipitation and inhibiting bacterial metabolism and growth (Karanasios et al, 2010; Khin & Annachhatre, 2004).

In the results below, it is observed that activated sludge (RAS) maintained a stable pH within desired range of 7.5 as shown in Figure 3-3, so as Type II methanotroph and AOB pure culture. While for biomass coming from anaerobic digestion (AD) and Type I methanotrophs, pH increased up to 9-9.5, which is not favorable for hydrogenotrophic denitrification process, and indicates an unstable environment for microbial growth and activity.

On the other hand, a different trend was discovered in terms of alkalinity, as it was in favor of production, indicating proper denitrification and nitrite removal, only in the mixed cultures; RAS and AD. While alkalinity went in negative direction and was consumed by the bacteria in the pure cultures, AOB and methanotrophs as seen in Figure 3-4.

By observing these results combined, it is concluded that optimum operating conditions discussed in literature and desired outcomes were achieved using activated sludge culture (RAS), where stable pH was maintained and alkalinity was produced with around 30%.

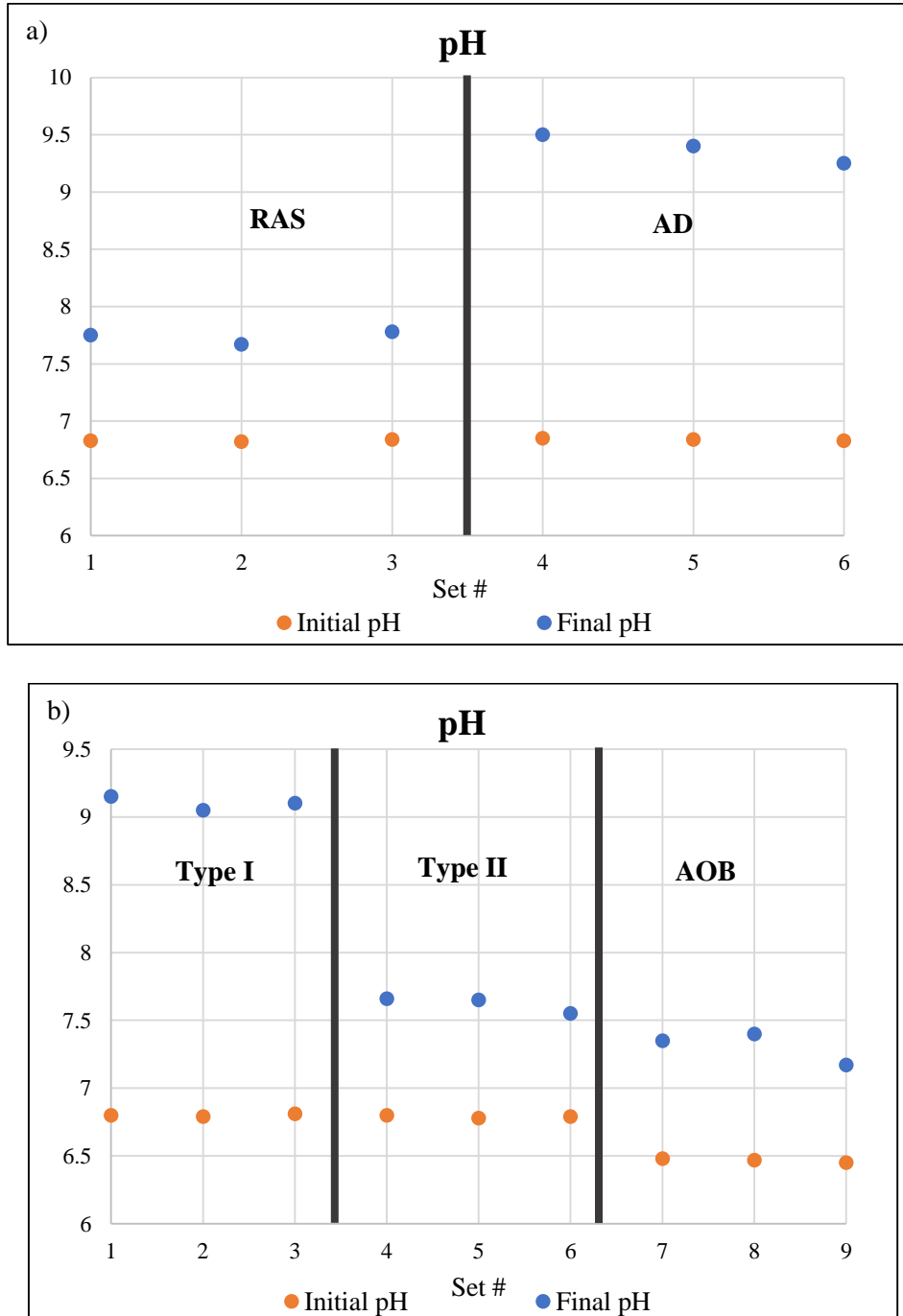


Figure 3-3 pH profile for the 5 different cultures: a) experiment 1 conducted for cultures coming from mixed nature, b) experiment 2 for cultures coming from pure reactor

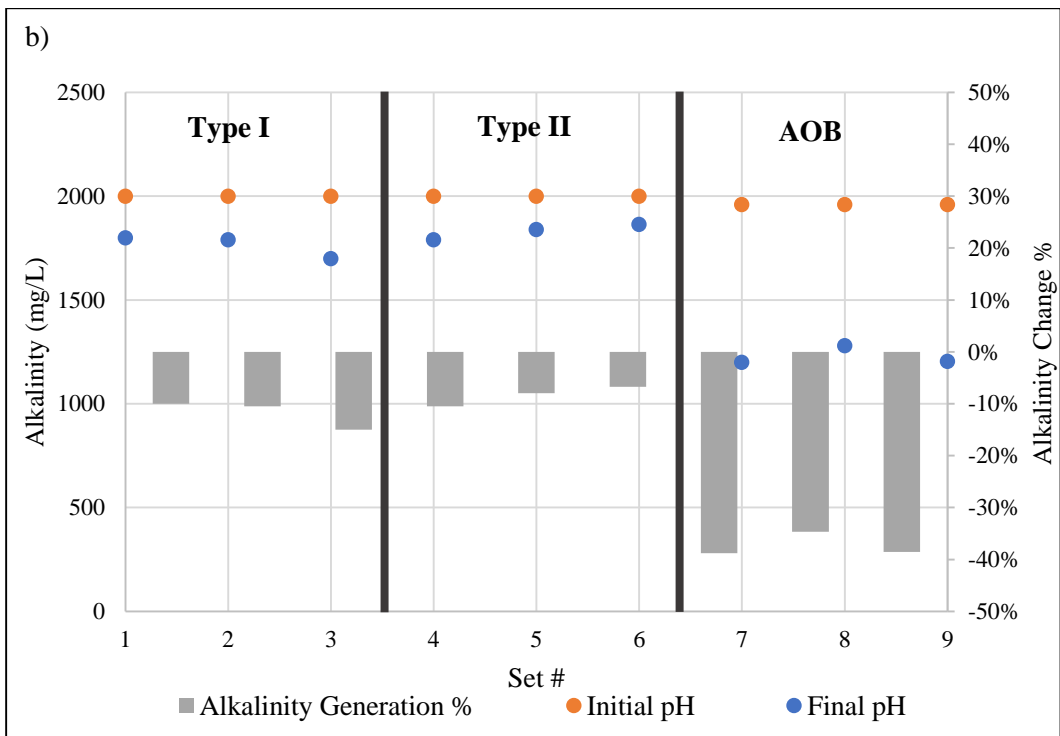
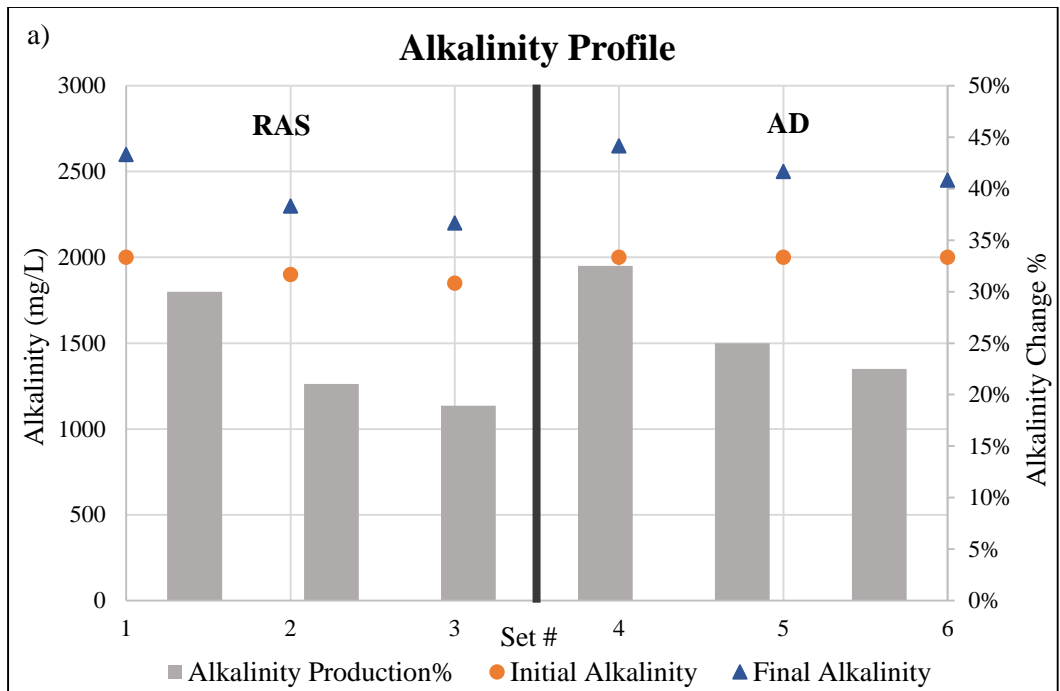


Figure 3-4 Alkalinity profiles and production efficiency for 5 different cultures: a) mixed cultures, b) pure cultures of Methanotrophs and AOBs

3.3.4. Microbial Community Analysis

Four types of biomass were investigated after the experiments were done, however the fifth sample which had the AOB culture was inadequate for analysis. In the figure below, the classification of the cultures according to the dominant phylae existing therein. For the four cultures, *proteobacteria* (>50%) and *bacteroidetes* (>30%) were the dominant phylum. For the activated sludge culture, it can be seen that there's a limited number of phylum strains which shows the selectivity in the RAS nature. Studies on hydrogenotrophic denitritation or denitrification have involved a limited bacteria species, due to the fact that a hydrogenotrophic denitrifying environment is highly selective. These organisms must have the capacity to utilize nitrite as electron acceptor, grow with inorganic carbon under anoxic conditions, and utilize H₂ as electron donor.

Genus level classification is important when it comes to microbial communities, as it identifies the dominant genera in a specific reactor, hence leading to focusing on certain strains behavior, and understanding their growth characteristics.

Thermomonas genera is abundant throughout the activated sludge (RAS) batch and anaerobic digestion (AD) batch with ~20% as seen in Figure 3-5. This genera was isolated in numerous previous denitrifying reactors, mainly they have mesophilic nature and reduce nitrates to nitrites and into nitrogen eventually using autotrophic carbon sources (Mergaert et al, 2003; Straub, Schönhuber et al , 2004; Xing et al, 2016).

As for Type I methanotrophs, *Pseudomonas* and *Macellibacteroides* bacteria were abundant in the sample with 19% and 18% each respectively. While for Type II methanotrophs, some common methanotrophs such as *methylocytis* (14%) and *chrysobacterium* (27%) were the most abundant across the sample. For both cultures, no common or known denitrifier was found in their

samples, rather only methanotrophic and anaerobic cultures were present (Davies, 1973; Lindner et al., 2007; Morris et al., 2002). Therefore, it is deduced that these cultures do not have species capable of performing denitrification using hydrogen, which is confirmed by their low efficiency in nitrite removal.

These results confirm that the RAS and AD as mixed cultures have more denitrifying potential and could be utilized in autotrophic denitrification reactors.

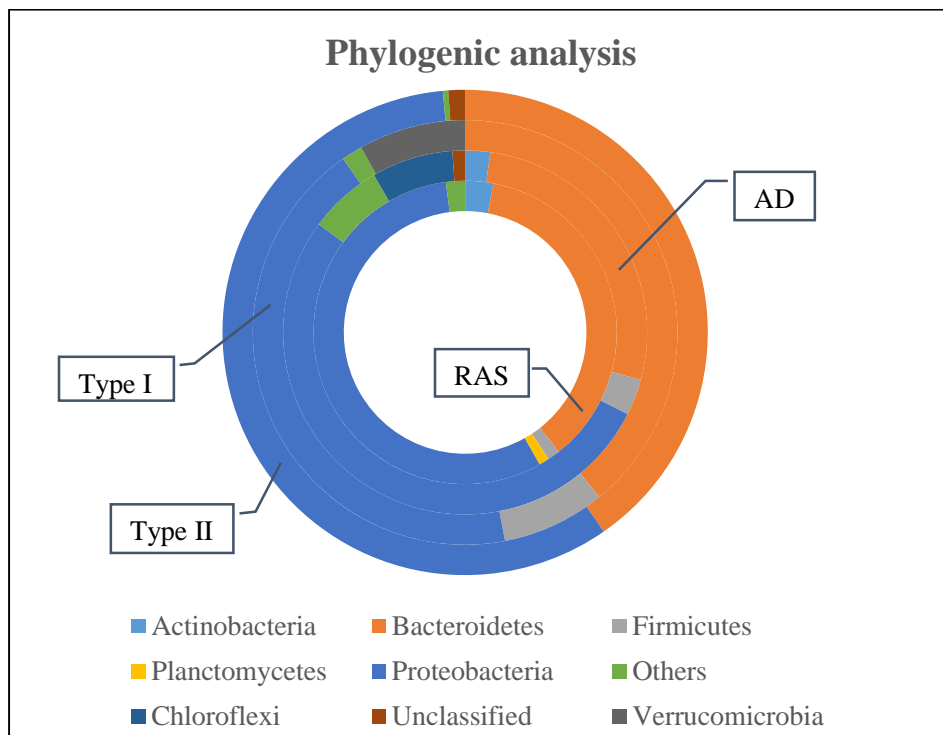


Figure 3-5 Phylum classification across the cultures

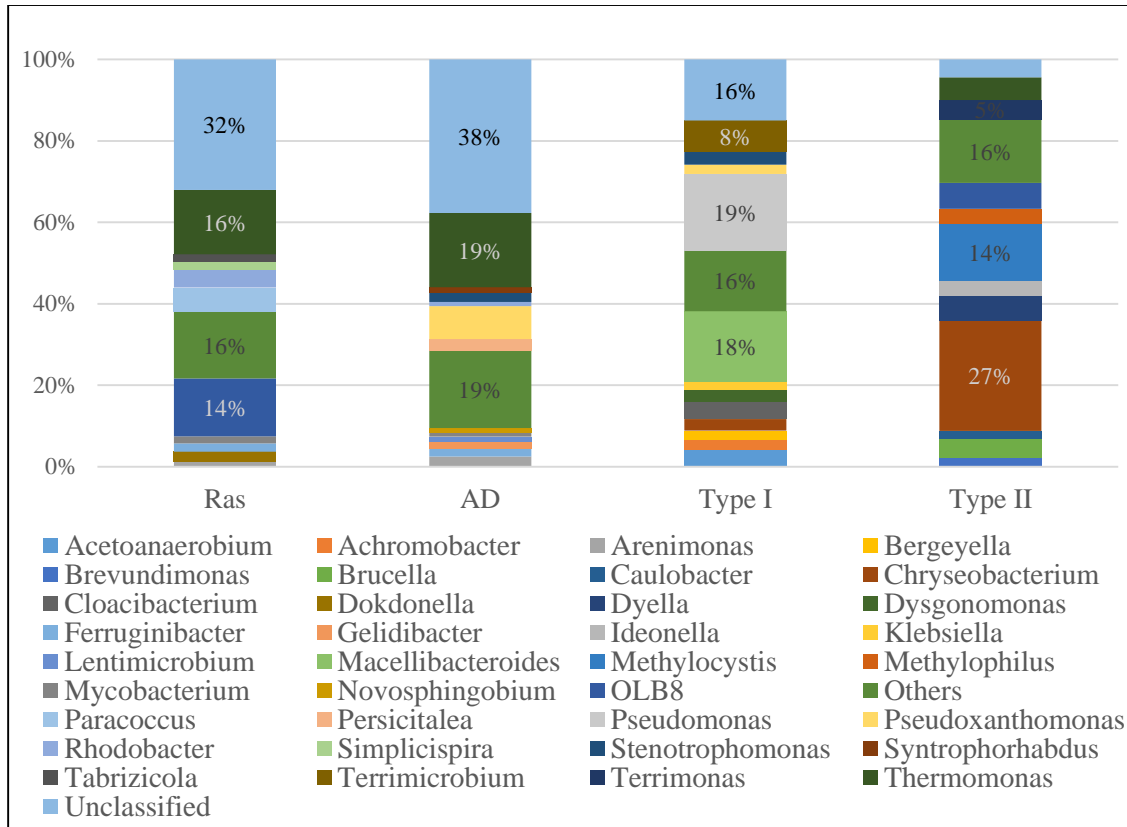


Figure 3-6 Microbial classification of the cultures according to genus

Based on the previous findings, it is evident that when it comes to nitrite removal, mixed culture media is more efficient since activated sludge (RAS) having the highest potential of hydrogenotrophic denitrification.

It was also found in regard to operating parameters that alkalinity and pH were maintained in a stable level in the presence of mixed culture, specifically RAS, more than in pure culture bacteria as AOB and methanotrophs.

Microbial analysis results confirmed that the dominant genera in mixed cultures, *Thermomonas*, belong to denitrifying bacteria that can reduce nitrogenous oxides to nitrogen gas. This confirms the performance of RAS which was more successful in achieving complete denitrification in HRT of 4 days with efficiency higher than 90%.

3.4. Conclusion

Several literature and previous research have been carried out to investigate the process of hydrogenotrophic denitrification. However, employing hydrogen as electron donor in denitrification, where nitrite is the electron acceptor instead of nitrate, is a novel process that needs thorough research. In doing so, identifying the proper microbial culture that could have potential in treating high nitrite levels in wastewater is crucial.

An experiment was designed that studies autohydrogenotrophic denitrification in five different microbial cultures coming from different sources and processes. The main control parameters were kept constant across all batches, such as pH, alkalinity, and nitrite concentrations.

It was found that RAS, which is a mixed culture from activated sludge system, had a significant nitrite removal in presence of hydrogen gas, and maintained all other operating conditions within stable and desired range. The nitrite removal efficiency reached 90% in the batch that was inoculated with RAS as biomass.

The process needs optimization and deep investigation into the genus and strains capable of reducing nitrite in the wastewater and utilizing hydrogen gas selectively. However, it can be deduced that mixed culture utilization is proven to be more feasible and in favor of autohydrogenotrophic denitrification as found in the previous studies and research.

Chapter 4 Development of Hydrogenotrophic Denitrification for Municipal Wastewater under different F/M ratios

4.1. Introduction

Side-stream wastewater has high concentration of nutrients and pollutants, with a lot of potential for resource recovery and waste to energy production. This has driven research into studying the potential of more efficient and energy saving technologies in treating wastewater, with the objective of producing alternative energy paths and resources from it.

The study and optimization of hydrogenotrophic denitrification and the parameters affecting the process have been sought by research in the recent years. From the main advantages of this method are low cell growth, no need for post treatment as there is no byproduct from the process, and most importantly is the cleanliness of the process and its harmlessness to humans, except for minor safety concerns due to use of hydrogen gas which has low solubility and might create an explosive atmosphere if not controlled properly (Karanasios et al, 2011; Park & Yoo, 2009).

However, this concern is easily overcome by ensuring the provision of sufficient amounts of carbon source, substrate, biomass and completely mixing and diffusing the gas inside.

As previously discussed, there are many parameters to control in order for successful treatment and nitrite removal. Many optimization techniques have been employed into the process such as limiting carbon source or pH control as done by (Ghafari, Hasan, Aroua, 2009) or by controlling influent nitrogen concentration (Vasiliadou, Pavlou & Vayenas, 2006).

For this current research, one of the parameters that is observed to have an impact during running the experiments was food to microorganism (F/M) ratio. F/M indicates the amount of solids and biomass content in terms of MLVSS versus the loading capacity of the substrate in the

wastewater, which is nitrite in this case. The higher the F/M ratio, the lower the biomass density and solids in regards to the substrate. Throughout this paper, the effect of F/M ratio along with alkalinity and nitrite concentrations on operation of autohydrogenotrophic denitrification is assessed by conducting different batches while changing their F/M ratio, and monitoring their nitrite reduction performance along with their operating conditions such as pH and alkalinity to maintain stability and cell growth.

4.2. Materials and Methods

4.2.1. Experiment Design

The same setup as the experiment explained in the previous section is used for this stage as shown in the figure below.

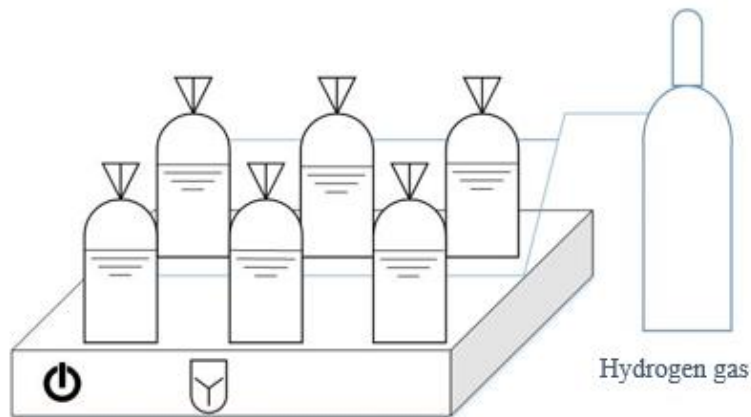


Figure 4-1 Schematic diagram of the batch experiments

According to (Karanasios, Vasiliadou, Pavlou, 2010), alkalinity, hydrogen and nitrite concentration are critical parameters affecting the performance of hydrogenotrophic denitrification. Therefore, during this stage of fed batch experiments alkalinity concentration was controlled and different concentrations were selected through different experiments. To study the

change in based on the relation between nitrite concentrations and biomass density, this stage consisted of three experiments with different influent parameters as shown in Table 4-1.

Table 4-1 Experimental Setup and influent parameters

Experiment	#1		#2		#3		#4	
Nitrite (mgNO₂-N/L)	400		400		400		800	
H₂ Dose (mL)	50							
Alkalinity (mgCaCO₃/L)	1000	2000	1000	2000	2000	1000	2000	
F/M	0.15		0.15		0.4		0.3	

4.2.2. Hydrogen Sparging

Refer to Section 3.2.2 for details on hydrogen gas sparging method and mixing technique.

4.2.3. Synthetic Feed

The synthetic feed used for this experiment is illustrated in Section 3.2.3. In this experiment, two nitrite concentrations were used to study the effect of changing the feed concentration in terms of nitrite in relation to biomass density known as F/M ratio.

4.2.4. Biomass and Seed

The biomass used for these experiments had different solids content, in order to fulfill the objective of different F/M ratio. This is done by changing the nitrite concentration or mixed liquor suspended solids. In this study, the recycled activated sludge used had low initial solids content. It started with total suspended solids (TSS) and volatile suspended solids (VSS) of 10

and 5 g/L respectively. This led to an F/M ratio of 0.4 gN/gVSS. After dewatering the sludge, suspended solids in the thickened sludge were 14.5 gVSS/L, to reach a food-to-microorganism ratio (F/M) of 0.15 gN/gVSS.

4.2.5. Analytical Methods

Specific information on analytical instruments, and details on methods used are presented in Section 3.2.6.

4.3. Results and discussion

4.3.1. Batch Performance

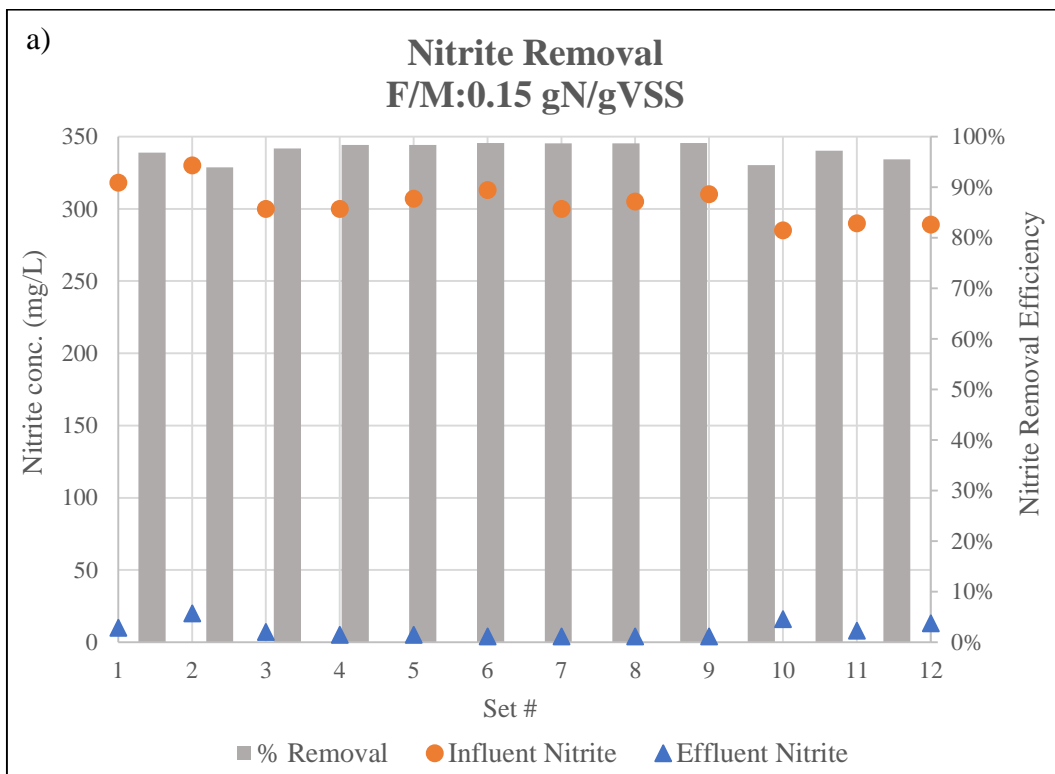
For this set of experiments, it was run on a number of phases. Through each phase a certain parameter was changed in order to optimize the performance, and measure the potential of mixed culture to treat the wastewater. Mainly, alkalinity was targeted as the parameter to be optimized through these experiments, while keeping all others constant. One of the parameters associated with alkalinity directly is pH, which was monitored as well. Secondly, food to mass (F/M) ratio changed throughout these experiments, due to changing the biomass, activated sludge, during running the fed batches by getting fresh supply from the treatment plant.

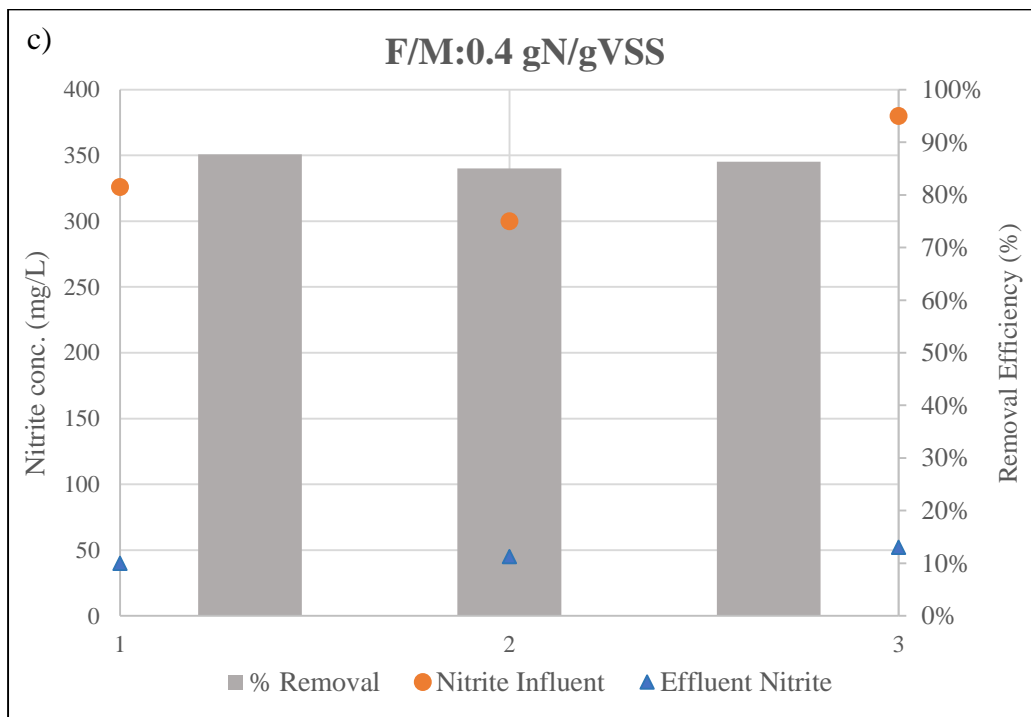
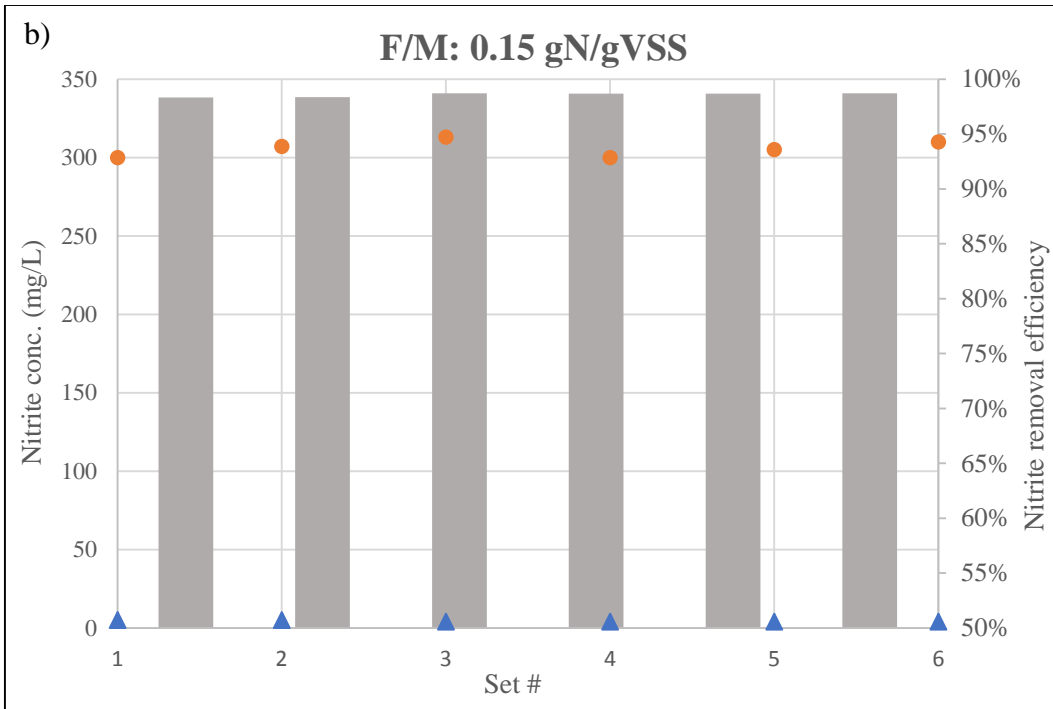
In a study conducted by (Ghafari, S; Hasan, M.; Aroua, 2009b), hydrogenotrophic denitrification of 20 mgNO₂-N/L was achieved using ranges of 1000 to 2000 mg/L alkalinity at a rate of 25 mgNO₂-N/gVSS/h.

4.3.2. Nitrite Removal Rate

In the conducted experiments, nitrite reduction was observed with efficiency up to 97% ± 2 for the first two experiments and 87% ± 2 for the 3rd experiment which involved one set of triplicates as shown in the charts below.

Keeping alkalinity constant, it is observed that when influent nitrite concentration was set to 400 mgNO₂-N/L, as for experiment 1-3, the nitrite removal efficiency is high and exceeds 90%, making the final nitrite concentration less than 10 mgNO₂-N/L, which is a really low concentration for side stream wastewater with high nutrient concentrations. While in experiment 4, which comprised of 2 runs having 800 mgNO₂-N/L, the nitrite removal efficiency dropped to below 50%, with nitrite concentration reduced to 300±30 mgNO₂-N/L.





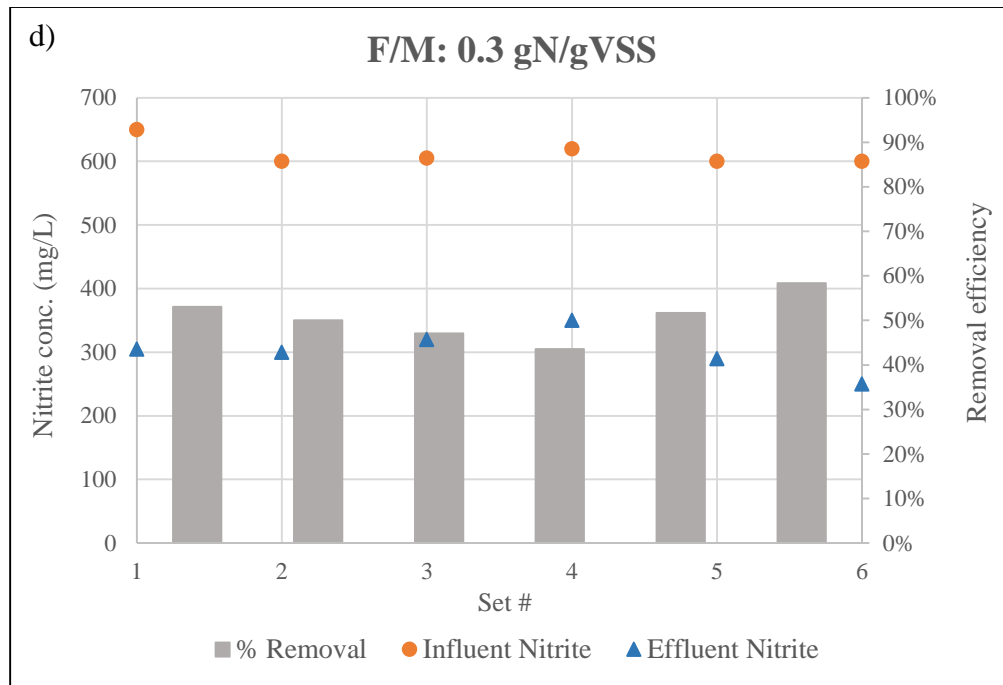


Figure 4-2 Nitrite removal profile for: a) experiment 1, b) experiment 2 of F/M 0.15, c) experiment 3 with of F/M 0.4 gN/gVSS, d) experiment 4 with 800 mgNO₂-N/L, F/M: 0.3 gN/gVSS

4.3.3. Effect of food to microorganisms (F/M) ratio

In this phase the initial F/M was set to 0.15 given initial biomass concentration of 14.5 gVSS/L after thickening. Later on during operation, experiment 3, F/M was increased to 0.4 by using biomass of 5 gVSS/L, in order to study the effect of changing F/M ratio on nitrite removal. In experiments 1-3, influent nitrite concentration was selected to be 400 mgNO₂-N/L. Overall, the nitrite removal rate for 1st two experiments exceeded 95% as seen in Figure 4-2, while for 3rd experiment it went up to 85%. As for the biomass growth and growth yield, an increase was noticed in the cell growth yield in lower F/M ratio, by 5%. As for the higher F/M ratio, the growth yield only increased by 1%. Further investigations are required to clarify such observation, but it can be initially illustrated that the lower F/M ratio, corresponds to higher nitrite removal and growth yield.

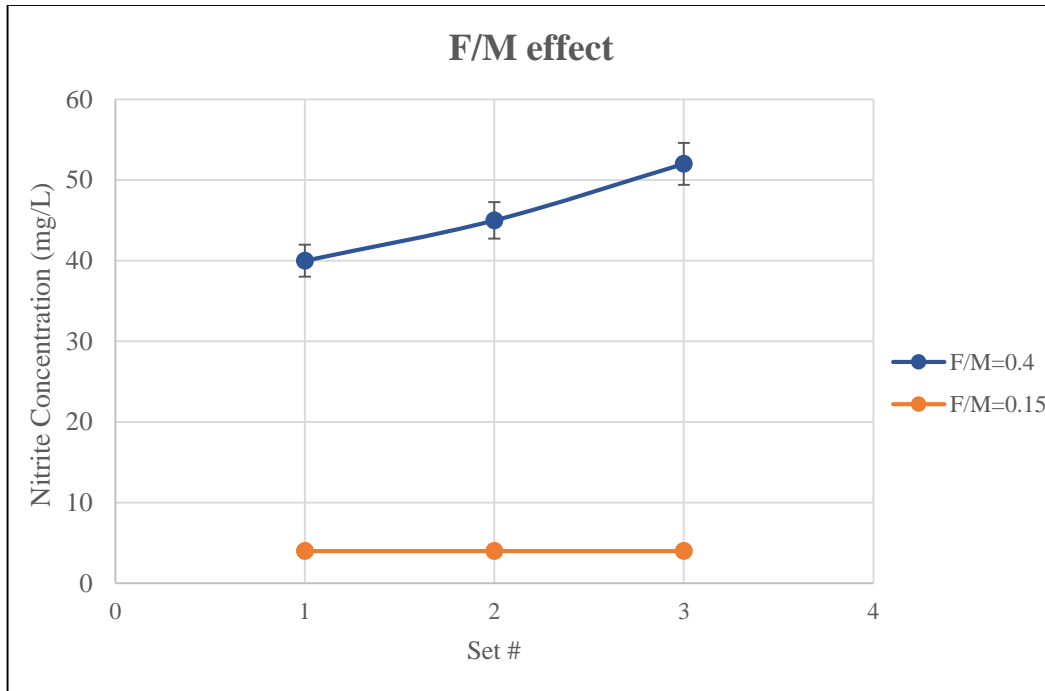


Figure 4-3 Effect of F/M ratio vs Final nitrite concentration, Orange and blue curve refer to Experiments 2 and 4 respectively

4.3.4. Nitrite Uptake Rate

Generally, highest nitrite uptake rate was 3.23 mgNO₂/hr for concentration of 400 mgNO₂-N/L, corresponding to alkalinity and F/M ratio of 1000 mgCaCO₃/L and 0.15 respectively. As for nitrite concentration of 800 mgNO₂-N/L, the highest uptake rate was 3.65 mgNO₂/hr corresponding to alkalinity of 2000 mgCaCO₃/L and 0.15 F/M ratio.

Based on the previous findings, it is evident that high potential of hydrogenotrophic denitrification exists in mixed culture medium with the presence of the right parameters. Optimization of the process lies herein by combining all required parameters in a single reactor, in that case alkalinity, F/M ratio and hydrogen dosage are key elements for the success of the process. It is confirmed that 2000 mg/L alkalinity is able to achieve removal of 400 mg/L nitrite with efficiency as high as 95%, with uptake rate of about 3.2 mgNO₂/hr. The effect that F/M ratio has

on mixed culture performance is of significant difference, since lower biomass densities with lower solids content, increased the efficiency by approximately 10%. This is considered as a more economic and achievable approach.

4.4. Conclusion

Hydrogenotrophic denitritation is a novel process that employs hydrogen gas and mixed culture of microorganisms in reducing nitrite that exists in wastewater, specifically side streams that have high concentration of pollutants and nutrients which needs advanced treatment and processing. It was concluded that at STP, and at 40% volume occupied with hydrogen gas hydrogen, Nitrite removal is highly efficient using mixed culture bacteria in presence of inorganic/autotrophic carbon source, sodium bicarbonate. The parameters investigated in this chapter were mainly alkalinity source and F/M ratio, which is an indicator of biomass density. In the next stage of the experiment, the optimum values evaluated here which are 2000 mgCaCO₃/L and 0.15 F/M are going to be operated with different parameters to reach the most feasible conditions for this process.

Chapter 5 Influence of Nitrite to Microorganism (N/M) and Hydrogen to Microorganism (H/M) Ratios on Hydrogenotrophic Denitrification of Municipal Wastewater

5.1. Introduction

The study and optimization of hydrogenotrophic denitrification and the parameters affecting the process have been sought by research in the recent years. From its main advantages are low cell growth, no need for post treatment as there is no byproduct from the process, and most importantly is the cleanliness of the process and its harmlessness to humans, except for minor safety concerns due to use of hydrogen gas which might create an explosive atmosphere. (Park & Yoo, 2009). However, this concern is easily overcome by ensuring the provision of sufficient amounts and completely mixing and diffusing the gas inside.

As previously discussed, there are many parameters to control in order for successful treatment and nitrite removal. Many optimization techniques have been employed into the process such as limiting carbon source or pH control as done by (Ghafari, Hasan, Aroua, 2009b). Throughout this paper, both H/M and N/M ratios were changed for optimization purposes according to the change in the biomass density. Therefore, their influence on the microbial activity and operation of the process had to be investigated. Different nitrogen concentrations were added to cultures with the same biomass density.

5.2. Materials and Methods

5.2.1. Experiment Setup

Refer to Section 3.2.1 for the specific materials used for this experimental setup. For calculation of N/M ratios for the purpose of this study, the nitrite concentration in the wastewater was divided by the VSS of biomass used. While for H/M ratio, the hydrogen dosage was converted to concentration using hydrogen gas density at STP, then divided by VSS to get the ratio for different dosages. During running different phases of the experiment, hydrogen dosage and influent nitrite concentrations were changed a number of times for optimization purposes. The effect of changing them was studied and reported by looking at certain ratios which were, Hydrogen to Microorganism ratio (H/M) and Nitrite to Microorganism ratio (N/M). Hydrogen concentration was calculated by converting the volumetric dosage in milliliters. This stage consisted of four experiments which consisted of different alterations and scenarios as shown in Table 5-1.

Table 5-1 Experimental Setup and influent parameters

Experiment	#1			#2		#3		#4
Nitrite (mgNO₂-N/L)	400			400		800		400
H₂ Dose (mL)	50			25		50		100
Alkalinity (mgCaCO₃/L)	500	1000	2000	500	1000	1000	2000	1000
H/M	0.002			0.002		0.003		0.005
N/M	0.03			0.04		0.07		0.03

5.2.2. Hydrogen Sparging

Since the hydrogen dosage was altered for this set of experiments, the headspace was vacuumed from air and injected with the selected hydrogen amount after adding the synthetic wastewater, which were 25, 50 and 100 mL equivalent to 20%, 40% and 80% respectively of the sample volume. Details on injection method and tools used are found in Section 3.2.2.

5.2.3. Synthetic feed

The synthetic feed used for this experiment is illustrated in Section 3.2.3. In this experiment, two nitrite concentrations were used, in order to investigate the relation between changing hydrogen and nitrite concentrations. Bicarbonate concentrations (i.e. carbon source) concentrations within each batch were altered to monitor the effect on alkalinity profile during operation.

5.2.4. Biomass and Seed

The biomass used to inoculate the batch was waste activated sludge (WAS). The activated sludge was freshly collected from Humber wastewater treatment plant, Ontario, Canada.

The activated sludge was preserved in a cold room at 4°C before running the experiment. Its total suspended solids (TSS) and volatile suspended solids (VSS) were found to be 14 and 10 g/L respectively. After dewatering the sludge, suspended solids in the thickened sludge was 14.5 gVSS/L.

5.2.5. Analytical methods

Samples were taken from influent (before starting experiment) and effluent stage (on a day to day basis), and collected in airtight bottles daily, and refrigerated at 4°C until analysis was conducted. Specific information on analytical instruments, and details on methods used are presented in Section 3.2.6.

5.3. Results and Discussion

5.3.1. Overall Performance

For this set of experiments, it was run on several stages. For each stage, two ratios were coupled and altered for each experiment to optimize the amount of hydrogen and nitrite concentration versus suspended solids found in the biomass, hence leading to a better performance.

In doing so, alkalinity and pH were kept constant in comparing different experiments, afterwards the effect on pH resulting from these alterations would be studied. High potential in denitrification using hydrogen gas was achieved by coupling the proper H/M and N/M ratios.

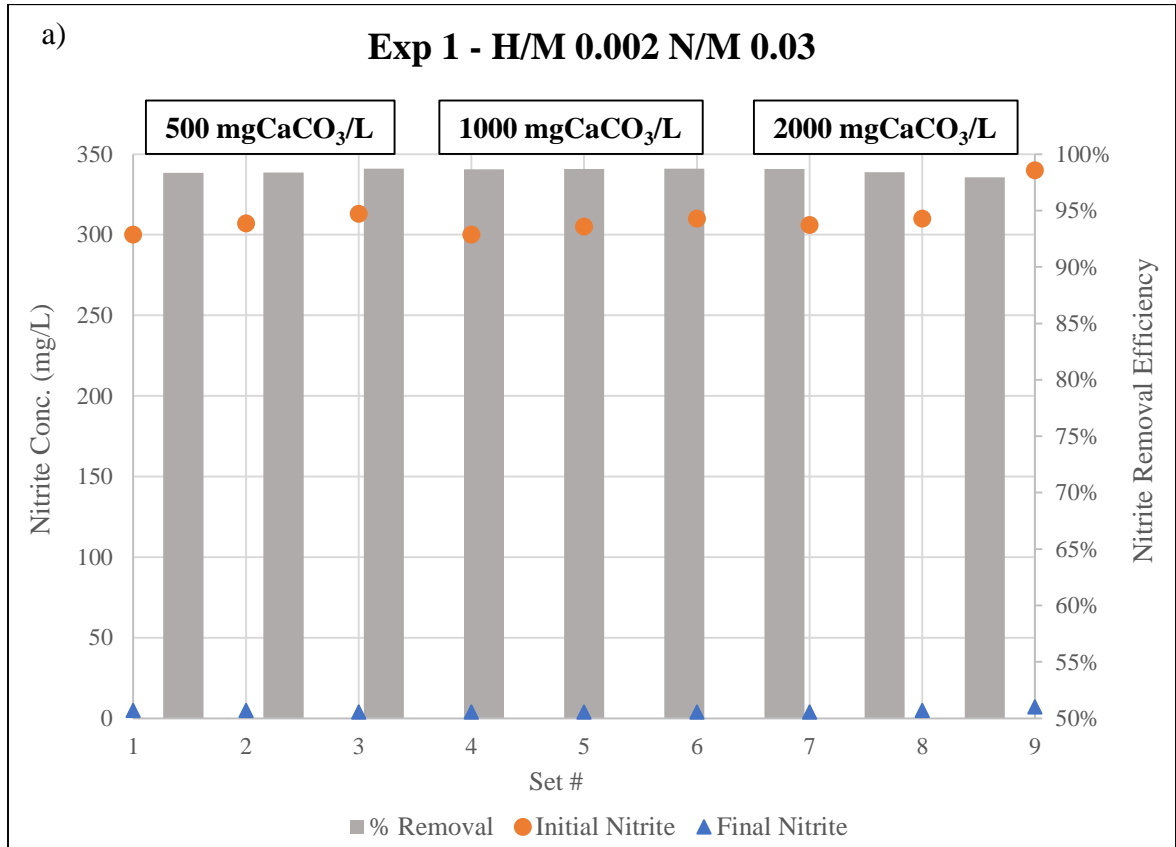
Due to its novelty, there's a lack in literature and previous research attempts for this process, hence more studies and experiments are needed to confirm and validate these results.

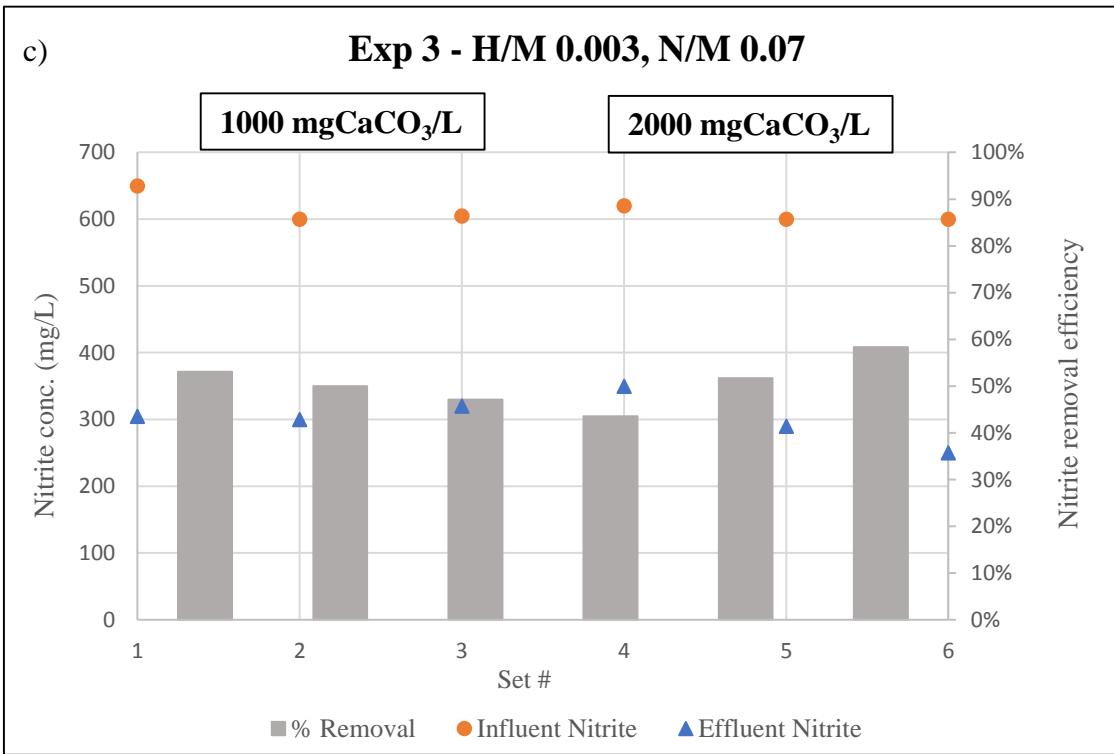
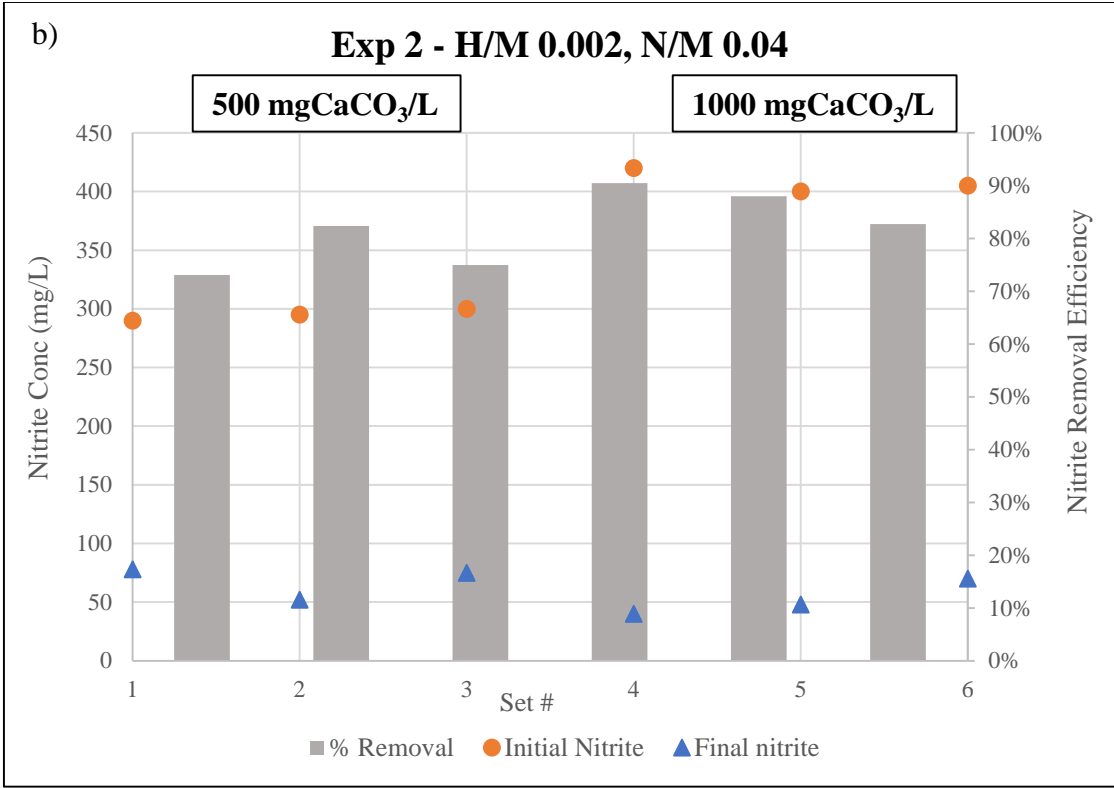
5.3.2. Nitrite Removal Efficiency

It is observed from the conducted experiments' results that nitrite removal was achieved with high rates for the different H/M and N/M ratios, with some variation in the efficiency of each and overall performance.

For the 1st experiment which had the lowest combination of H/M and N/M ratios, 0.002 and 0.03 respectively, it recorded the best results in regards to nitrite removal efficiency as an overall efficiency of $97\% \pm 2$ was observed in all the samples. When compared to experiment #4, where N/M was the same, but H/M was increased by 60%, the removal efficiency dropped to less than 50% with nearly half removal efficiency. Results for Experiment #3 were comparable to that of #4 with similar nitrite removal efficiency around 50%, although N/M ratio was much higher for this set of experiment as shown in the charts below (Figure 5-1). This could be tribute to: 1) the

insufficient hydrogen supply in comparison to the nitrogen loading for experiment #3, or 2) the excess hydrogen supply in comparison to the low microbial culture available in the wastewater.





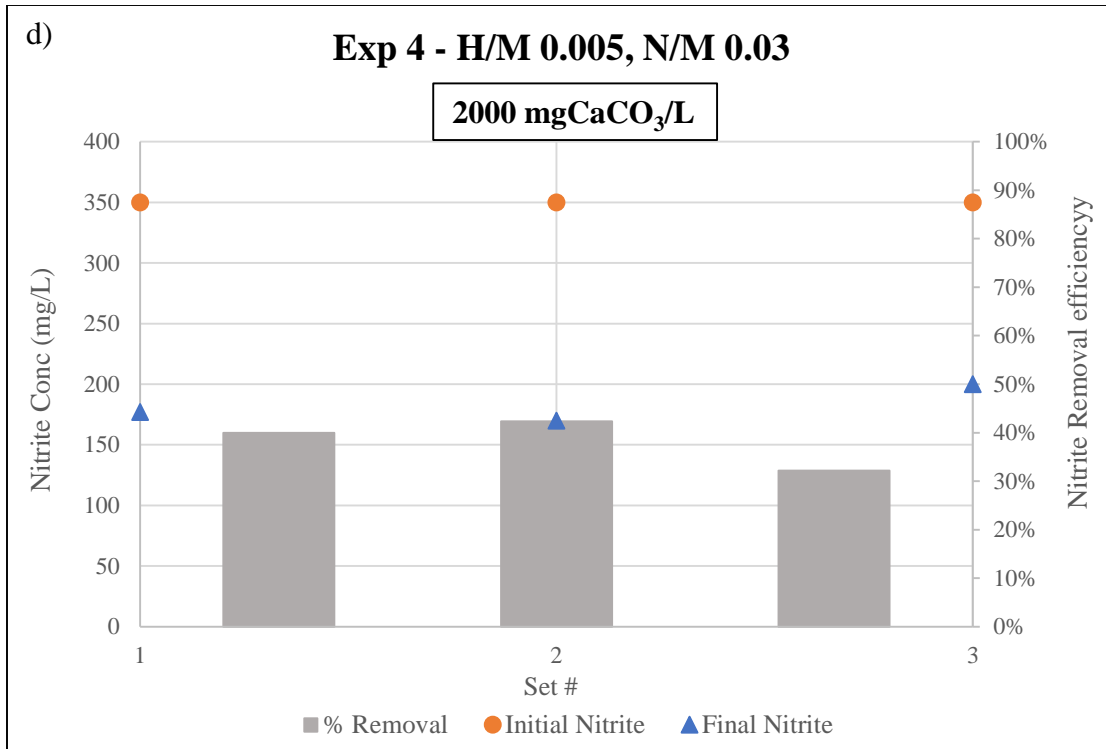


Figure 5-1 Nitrite removal profile for: a) experiment 1 (H/M: 0.002, N/M: 0.03), b) experiment 2 (H/M: 0.002, N/M: 0.04), c) experiment 3 (H/M: 0.003, N/M: 0.07), and d) experiment 4 (H/M: 0.005, N/M: 0.03)

In studying the effect H/M has on microbial activity and process overall performance, only batches with same influent alkalinity concentration are compared.

When first batch of experiment 1 and 2 are looked at, both having influent alkalinity of 500 mgCaCO₃/L, increasing N/M ratio leads to a decrease in the nitrite removal efficiency by 20% under same hydrogen supply. As for experiment 1 and 4, with alkalinity concentration 2000 mg/L, increasing the hydrogen supply beyond microbial requirements led to dropping the efficiency to 40%±2 with final nitrite concentration of 180±14 mg/L versus 5±1 mg/L for experiment 1 as shown in Figure 5-1.

5.3.3. Effect of H/M and N/M on pH

Results from different batches were compared in terms of pH to ensure the balance in the ecosystem, as pH is a dominant factor affecting the process and indicating the activity of the microbial culture.

According to (Lee & Rittmann, 2003) optimum pH for autohydrogenotrophic denitrification in a hollow fiber membrane setup was 7.7-8.6. Also, higher pH than that level can cause reverse reaction and nitrite accumulation is observed in the reactor (Karanasios, Vasiliadou & Pavlou, 2010).

To monitor the change in pH, it was done on two stages: 1) evaluating N/M ratios corresponding to same H/M ratio, 2) evaluating H/M ratios for similar N/M ratio. Hence, the results can be comparable and optimized.

The effect of the hydrogen to microorganism (H/M) ratio was found to be inversely proportional to pH. From the results below, it was observed that for first batch pH increased up to 7.5 for H/M ratio of 0.005, equivalent to 16% increase in terms of pH, while it increased up to pH 9 for H/M 0.002, around 38% as seen in Figure 5-2. Increasing the H/M ratio maintained a more stable pH level and bacterial metabolism for hydrogenotrophic denitrification. However, the lower H/M ratio of 0.002 still had acceptable pH levels when compared to the literature, especially for higher alkalinity concentrations. Further investigations are required, therein to clarify these observations.

After that, two experiments with the same hydrogen to microorganism (H/M) ratio of 0.002 were evaluated together. For the batch with higher N/M ratio of 0.04 there was a slight change in the triplicate samples of both experiments. This finding denotes that the effect of increasing N/M is minimal if within low range 10-20% as shown in Figure 5-3.

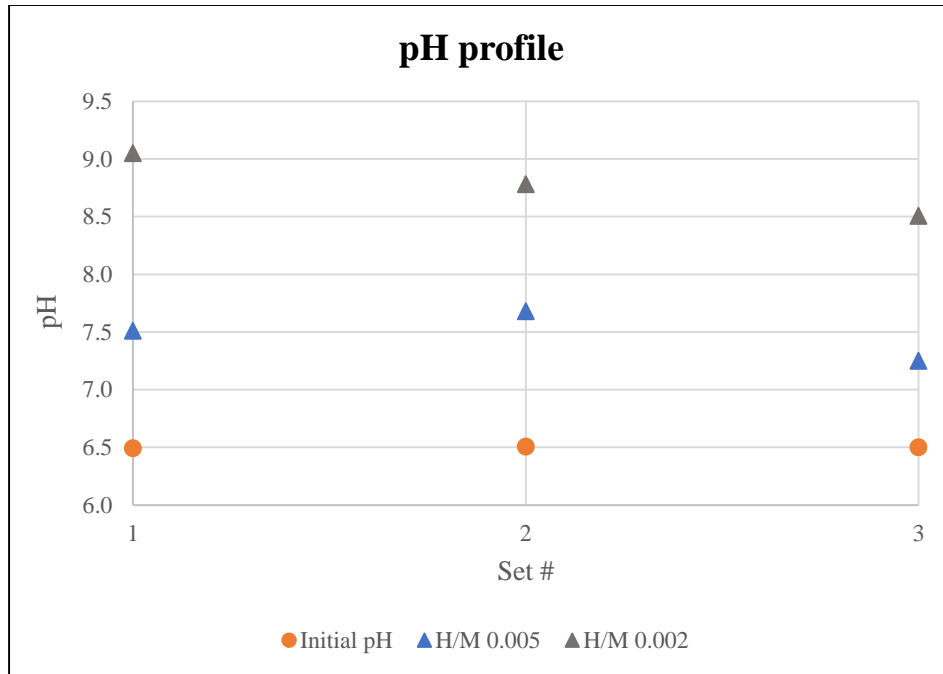


Figure 5-2 Change of pH with the different H/M ratios for N/M ratio of 0.03

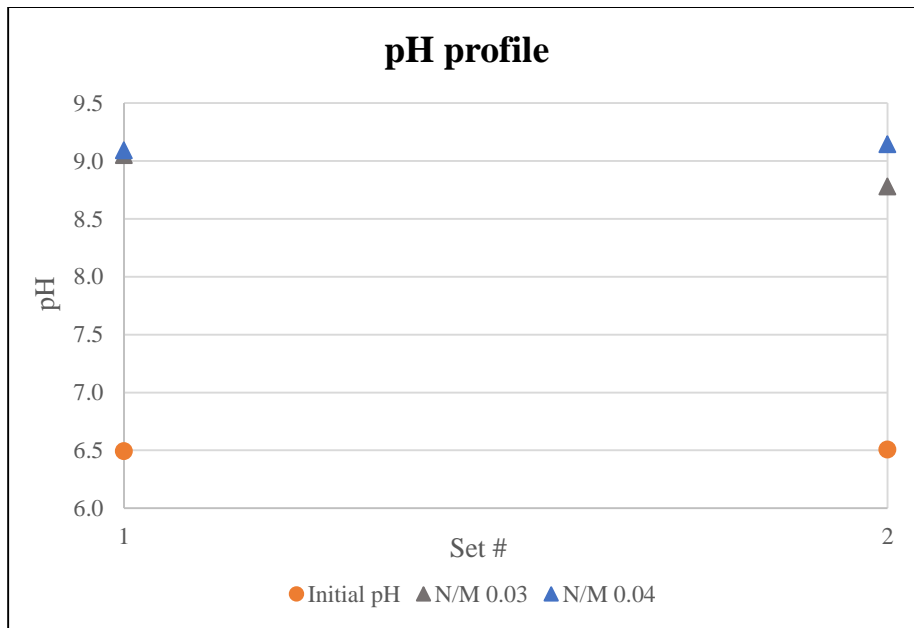


Figure 5-3 Change of pH with the different N/M ratios for H/M ratio of 0.002

Being the main inputs for the proposed approach, nitrite concentration along with hydrogen dosage require deep research and study. From the demonstrated results, it is deducted that excess

hydrogen supply translated to higher H/M ratio has a negative effect on nitrite removal efficiency as it dropped it from as high as 95% to lower than 50% removal for same given parameters. The same effect was also observed for overdosed nitrite loading and higher N/M ratios beyond bacterial capacity, which led to poor performance in the system as it decreased the efficiency of removal by 40%.

However, for the pH parameter which indicates the system stability and microbial activity, N/M ratio had minimal impact. As for the H/M ratio, it had an inverse effect on pH, as higher H/M ratio led to a lower increase in actual pH levels and maintained them within acceptable range.

5.4. Conclusion

Due to its novelty, hydrogenotrophic denitrification is a process that requires extensive investigation and research to prove its workability and benefits over other techniques. Since nitrite, and suspended solids (i.e. biomass) concentration along with hydrogen gas are form the main constituents of the process, the effect of coupling them was of crucial importance to the study. Therefore, a number of hydrogen to microorganism (H/M) and nitrite to microorganism (N/M) ratios were designed and altered together to validate results and optimize the parameters of the system. It was concluded that, the highest Nitrite removal efficiency occurred with H/M ratio of 0.002 and N/M ratio of 0.03 corresponding to 400 mgNO₂-N/L and 50 mL of hydrogen gas injected into the headspace. Coupling these low H/M and N/M levels from the projected results illustrated above would lead to optimizing the performance, leading to the ability to apply a more economic and resource friendly approach towards nitrogen removal from water resources.

Chapter 6 Development of Sequential Batch Reactor for Hydrogenotrophic Denitritation of Wastewater

6.1. Introduction

The increase of nitrogenous compounds observed in water resources is raising research concerns towards nitrate and nitrite removal to match global standard levels for drinking water. Due to its feasibility and advantages over other approaches such as chemical and physical treatments, biological denitrification is considered the most harmless, economic and environmentally friendly strategy. It is achieved by employing bacterial cultures that have denitrifying potential into wastewater with the presence of carbon source and electron donor, in which nitrogenous oxides act as terminal electron acceptor. When organic carbon compounds are used in the treatment process, it's known as heterotrophic denitrification. While inorganic compounds such as sulfur and hydrogen make the process autotrophic. (Karanasios & Vasiliadou, 2010)

When nitrite is utilized by microbial communities as electron acceptor, it is known as denitritation. The desired advantage from denitritation over denitrification (starting nitrate) is the less reaction time, as well as less energy source requirements. Moreover, facilitating the possibility of coupling the denitritation process with shortcut nitrification reactors (i.e. halting ammonia at nitrite oxidation stage), such as nitrite shunt or SHARON reactors, in order to have an integrated removal system of ammonia into nitrogen gas in one reactor.

In this section, hydrogenotrophic denitritation is aimed to be achieved in a SBR setup, in order to scale up the treatment capacity, decouple HRT from SRT to decrease the reactor volume and increase nitrite reduction rates. This process is autotrophic where hydrogen acts as an electron donor for the nitrite in the wastewater to convert it into nitrogen gas under the influence of the

responsible microbial strains. Since hydrogen gas (the electron donor/reactant) and nitrogen gas (product of the process) are both clean and harmless gases to the environment and humans, hydrogenotrophic denitrification was highly studied and adopted in the recent years, and considered as the best choice in comparison to other electron donors such as organic substrates and metals (Park & Yoo, 2009).

From several on hydrogenotrophic denitrification reactors and previous experiments, limited bacteria species were reported due to the highly selective nature of denitrifying environment, which requires the bacteria to be able to feed on nitrite as nitrogen source while acting as electron acceptor, and H₂ as electron donor. Also, these cultures should be able to utilize inorganic carbon source (such as bicarbonate) under anoxic or anaerobic conditions. (Karanasios & Vasiliadou, 2010)

For a sequential batch reactor (SBR) process, there needs to be more control on the operating parameters rather than batch system. This comes from the larger loading rates and more inoculum culture that may alter pH and alkalinity levels in the reactor. As well as that, some startup time is needed for acclimatization of the biomass culture, to allow for its growth and adapting to the specific conditions of the reactor such as temperature. SBRs became known as specific systems for nutrient removal, such as nitrification and denitrification since they can be modified to number of cycles, stages and each has a different design and application. Since denitrification is a temperature and pH sensitive process, these environmental factors need to be controlled and maintained within SBR for proper removal. (Ahn, 2006; Surampalli et al, 1997)

There's a lack of specific bacterial culture linked to this process in the literature to date. That's why a study of different bacterial cultures' ability to denitrify nitrite into nitrogen gas using hydrogen gas as electron donor is going to be carried out through this paper.

The effect of inoculum culture in up scaled system, specifically SBR, on the process of hydrogenotrophic denitritation is studied and illustrated, in order to identify the appropriate microorganisms that can be utilized in these reactors. A thorough investigation and optimization of the parameters and proper organisms is a desired outcome of the experiment.

6.2. Materials and Methods

6.2.1. Experiment Setup

For the purpose of this experiment which is up scaling and optimizing the process in hand, a sequential batch reactor (SBR) was designed. This process is usually used in wastewater treatment in order to decouple the hydraulic retention time (HRT) from solids retention time (SRT), hence reducing reactor volume and footprint, which leads to saving resources in terms of cost and energy.

The SBR was set up in a 2 L glass tight reactor (25 cm in height, 10 cm diameter) with an actual working capacity up to 1.7 L as seen below. The reactor was connected to a 5 L feeding tank, which contains the synthetic wastewater, using a peristaltic pump (Masterflex L/S Digital Pump System, Canada). An effluent tank (2 L volume) was also connected to be filled during decant phase with the same type of pump. This whole setup was operated and controlled using a control device known as New Brunswick BioFlo[®] 115 Benchtop Fermenter & Bioreactor, NJ as shown in figure below.



Figure 6-1 Glass bottles used for experiment

The reactor, feeding and effluent tanks were cleaned and autoclaved prior to the experiment to ensure the absence of any pathogenic activity.

In the suggested setup, the reactor was operating in two cycles per day, each cycle started with settling time for solids in the reactor for 20 minutes. Then, the wastewater was replaced and refilled in the fill/decant stage at the selected speed for 10 minutes simultaneously, before the reaction/mixing starts for 11.5 hours until the next cycle. The speed of the influent and effluent pumps was set to be 19 rpm, which allowed for 0.5 liters per cycle to be filled and decanted.

The biomass used throughout the experiment was acclimatized and preserved at 4°C cold room.

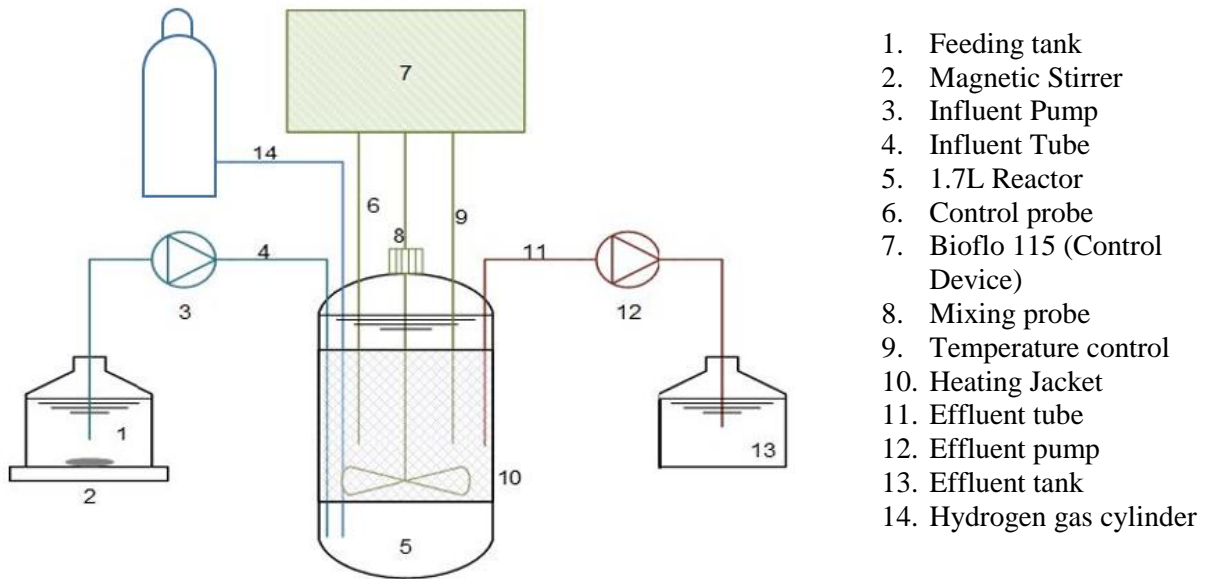


Figure 6-2 Schematic diagram of the reactor setup

6.2.2. Control Parameters

The influent parameters for the SBR setup were concluded mainly from the batch studies. It was also found by Ghafari et al. that hydrogenotrophic denitrification occurred at a sodium bicarbonate concentration of 2000 mg/L and pH of 7.5 (Ghafari, Hasan, Aroua, 2009b) as shown in Table 6-1.

Table 6-1 Experimental Setup and influent parameters

Experiment	#1
VSS	4 g/L
Nitrite Concentration	400 mgNO ₂ -N/L
H ₂ dose	1.75 L
Alkalinity	2000 mg CaCO ₃ /L
pH	6.5

6.2.3. Hydrogen Sparging

Hydrogen was sparged into the bottom of the reactor through a tube connected tightly to a hydrogen gas cylinder at a specific rate. This is to ensure the diffusion of hydrogen into the water not the headspace, due to its low solubility and safety concerns. It was injected for 10 minutes per day at a rate of 22 lph. Complete mixing was achieved by the agitation speed of the control device (Bioflo 115). This selected hydrogen volume was based on literature review and experiments performed earlier, with different hydrogen dosages in which this value had higher performance when employed with all other parameters.

6.2.4. Synthetic Feed

The reactor was inoculated synthetic wastewater with no organic substrate, which was freshly prepared and filled the feeding tank twice a week. The source of carbon in this experiment is sodium bicarbonate (NaHCO_3), it acts as alkalinity buffer as well for pH variations that occur during nitrite accumulation and denitrification process. A previous research attempted hydrogenotrophic denitrification (with nitrate) and used carbon dioxide and bicarbonate separately. It was proven that bicarbonate is better as it works towards stabilizing pH, which eliminates need for external buffer, as well as it doesn't produce intermediate harmful gases (Ghafari, S; Hasan, M.; Aroua, 2009a). The electron acceptor which is sodium nitrite is dissolved into deionized water containing potassium phosphate as phosphorus source, calcium chloride, and magnesium sulfate, as well as trace of mineral stock solution at a ratio of 1:0.001 per volume as shown in Table 6-2 to simulate nutrient rich wastewater. These salts and composition were selected as a result of the batch experiments conducted previously to confirm the concept.

Table 6-2 Synthetic wastewater composition

Compound	Concentration (per liter)	
NaNO₂	1971 mg	400 mgNO ₂ -N
NaHCO₃	3400 mg	2000 mg CaCO ₃
KH₂PO₄	106 mg	
CaCl₂	53 mg	
MgSO₄	42 mg	
Trace elements	1 mL	

6.2.5. Biomass and Seed

In this study waste activated sludge (WAS) was used to inoculate the reactor, since it was proven to be the most effective and potentially successful culture in achieving complete nitrite reduction using hydrogen gas from the fed batches conducted earlier in the study.

From the literature it was found that mixed cultures have better ability and selectivity for denitrification process, specifically hydrogen driven ones (Karanasios, Vasiliadou & Pavlou, 2010; Surampalli et al., 1997). Due to the lack of studies regarding hydrogenotrophic denitritation, mixed culture was employed in this reactor to confirm the findings from literature and batches conducted in this specific study.

The activated sludge (WAS) was dewatered and thickened to a high solids content of 4-5 gVSS/L. It is freshly collected from Humber wastewater treatment plant, Ontario, Canada.

The solids content and growth of biomass was monitored and measured by sampling throughout the experiment to ensure that cell growth is maintained.

6.2.6. Microbial Community Analysis

One sample was taken from the enrichment stage during the cultivation to ensure the dominance of denitrifying organisms in the recycled activated sludge mixed culture used. The sample was then sent to McMaster Genomics Facility, Ontario for microbial analysis and sequencing. Refer to Section 3.2.5 for specific method and kits used in the microbial analysis.

6.2.7. Analytical Methods

Samples were taken from influent (before starting experiment) and effluent stage (on a day to day basis), and collected in airtight bottles daily, and refrigerated at 4°C until analysis was conducted. Details on specific measurement methods and analysis are found in Section 3.2.6.

6.3. Results and discussion

6.3.1. Startup Period

In this experiment, the reactor described above was inoculated with the WAS biomass. For each experiment, the performance, and potential of the used culture to reduce nitrite was monitored and measured. Mainly, all parameters such as alkalinity, nitrite were kept constant to be able to have consistency in the performance of the reactor for a long period of time, and to have better control over the process.

The nitrite loading rate for the reactor (NLR) was 400 gN/m³d. This was fed over two cycles of fill/decant time 10 minutes. The starting activated sludge (WAS) had a solids content of 4500 mgVSS/L. The reactor was left for a week as a startup time to allow for cell growth and acclimatization of nitrite. Afterwards, daily hydrogen sparging began to monitor the reduction as illustrated hereafter.

6.3.2. Nitrite Removal Rate

In the conducted experiments, nitrite reduction was observed with the overall efficiency up to $91 \pm 8\%$, and the highest observed removal was 91% corresponding to final nitrite concentration of $5 \text{ mgNO}_2\text{-N/L}$. The specific denitritation rate was calculated to be $45 \pm 4.5 \text{ mgNO}_2\text{-N/gVSS/d}$. The performance of the reactor was overall consistent, but there were some irregularities in the behavior and removal pattern which were presumed to be due to some nitrogen loading shock that the bacteria was not able to sustain. Also, the sludge feeding methodology would need more optimization, as SRT was noticeably longer than HRT. One of the optimization techniques of this aspect is to reactivate the biomass inside the reactor by re-feeding an amount of fresh biomass mixed with the feeding wastewater, to replace an equal amount from the reactor on a regular basis. This would be confirmed after a kinetic study is performed for the process.

With alkalinity concentration of 2000 mg/L through the reactor, the nitrite removal rate of the reactor was $147 \text{ gN/m}^3\text{d}$. Keeping alkalinity and nitrite concentration constant, it is observed that when influent nitrite concentration was set to $400 \text{ mgNO}_2\text{-N/L}$, WAS adapted with a fast removal rate during 4 days and removed around 50% of the nitrite concentration. After that consistent performance was observed in the reactor with final nitrite concentration 35 mg/L .

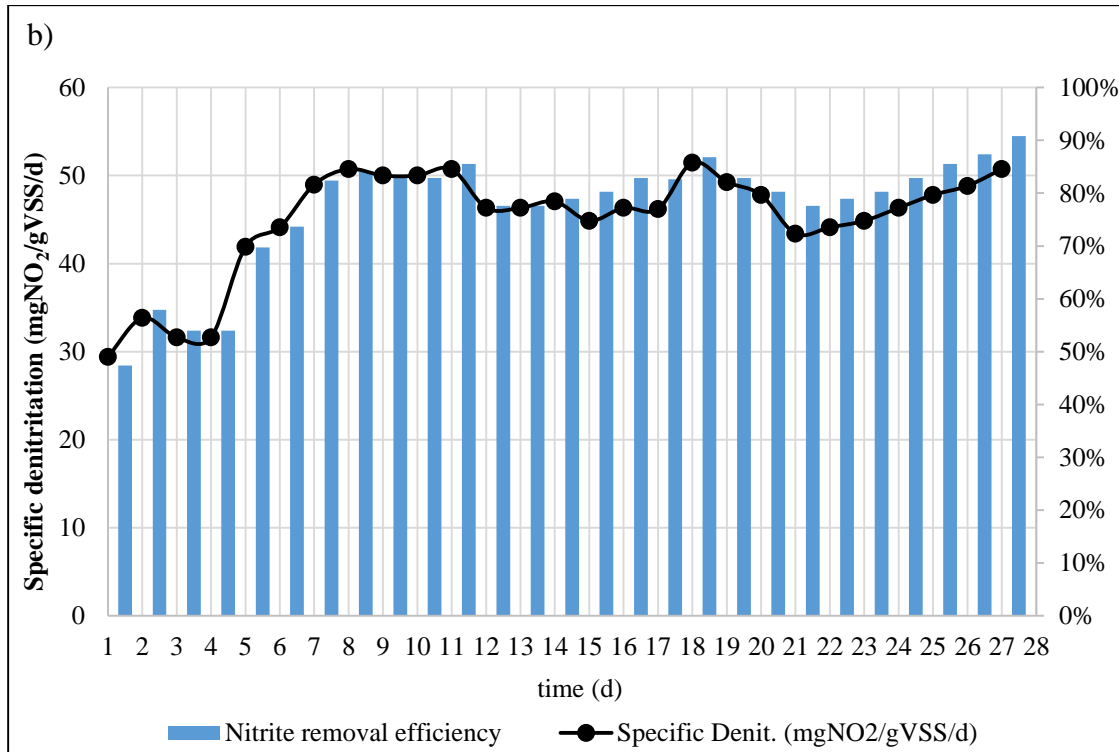
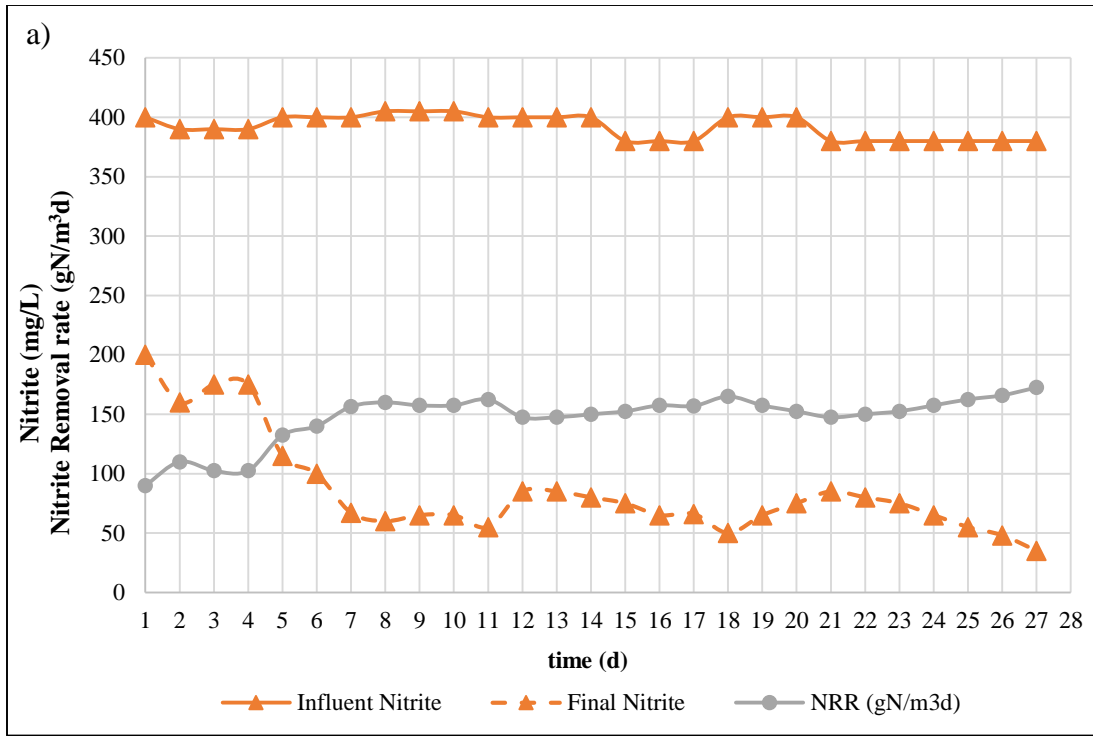


Figure 6-3 Profiles for: a) Nitrite levels and removal rate, and b) Specific denitritation rate and removal efficiency

6.3.3. Effect of denitritation on pH and Alkalinity

Alkalinity and pH were monitored as they are of the main parameters to measure the performance of hydrogenotrophic denitritation and bacterial activity and ensure stable reactor conditions. As for alkalinity, since denitrification as a whole process is in favor of producing alkalinity in terms of mgCaCO_3/L , therefore a measure of the potential of the mixed culture is an increase in alkalinity concentration of the wastewater but with reasonable limits so as not to inhibit cell growth. As for pH, the optimum pH levels for hydrogenotrophic denitrification to occur were found to be within 7.5-8.2. (Ghafari, Hasan, Aroua, 2009a; Karanasios, Vasiliadou, Pavlou, 2010)

The chart below shows the pH profile over the running of the reactor, it stayed within 7.5-8.3 which corresponds to literature values. Also, it indicates the activity of the bacterial culture since denitrification culture sustains within these ranges.

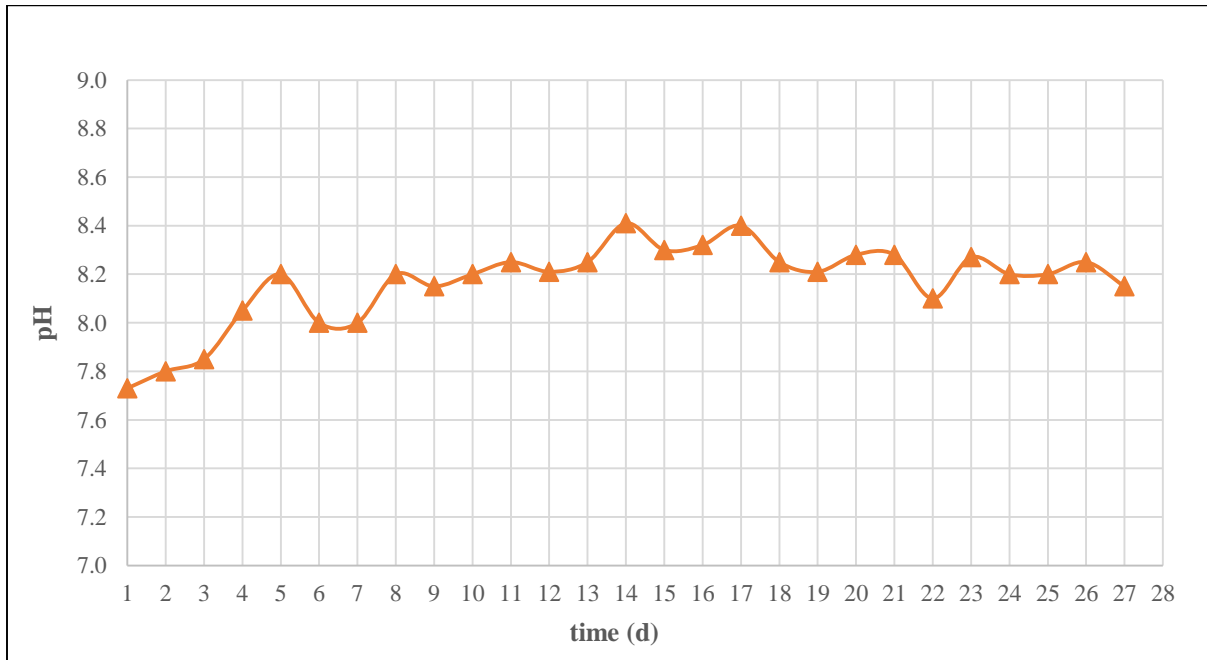


Figure 6-4 pH profile for the SBR reactor

As for alkalinity performance, the pattern was overall consistent and growing in terms of producing alkalinity. The reactor was enriched with 2000mg/L concentration which was deducted from batch experiments as the best performance value. The alkalinity increased until it reached 2900 mgCaCO₃/L corresponding to 45% within the reactor.

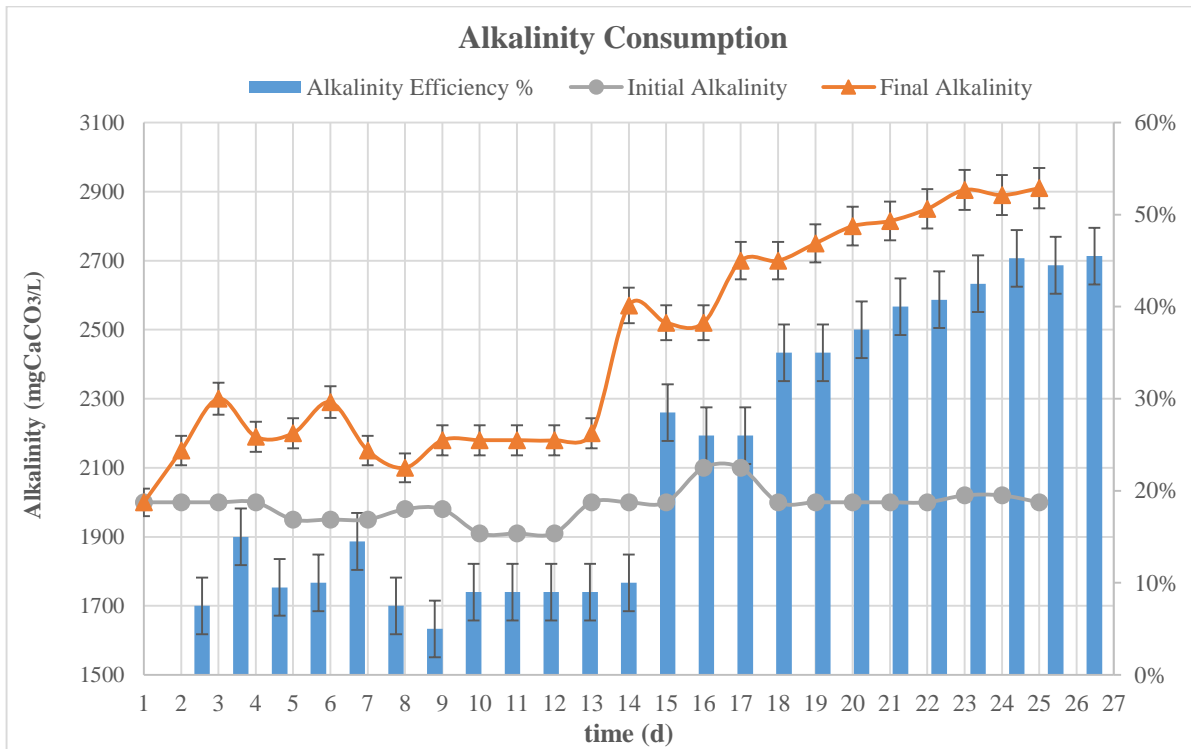


Figure 6-5 Alkalinity profiles and production efficiency

6.3.4. Microbial Analysis

There was a dominance from the *Proteobacteria* phylae, with almost 50% of the sample. This is supported by literature findings which confirm that this specific phylum is usually found in autotrophic denitrification reactors, with over 40% dominance (Ahn, 2006; Dasgupta, Wu, & Goel, 2017; Zhou et al., 2017). From the bacterial genus classification in Figure 6-7, the dominating micro-organisms are from the *Proteobacteria* phylum, and these are: *Alishewanella* bacteria (14.5%) which has the ability to utilize multiple electron acceptors for reducing nitrate

to nitrite, also considered an autotrophic denitrifier as it was isolated from autotrophic denitrification reactors. The other dominant genus is *Thermomonas* bacteria (12.1%) which is commonly found in and isolated from denitrifying reactors, it is classified as a mixed culture responsible for reducing nitrate to nitrite in these reactors. The ability to utilize nitrite is novel, hence not covered in literature, however these results confirm that mixed culture can overcome and sustain in denitrification reactors. (Mergaert et al., 2003; Xing et al., 2016)

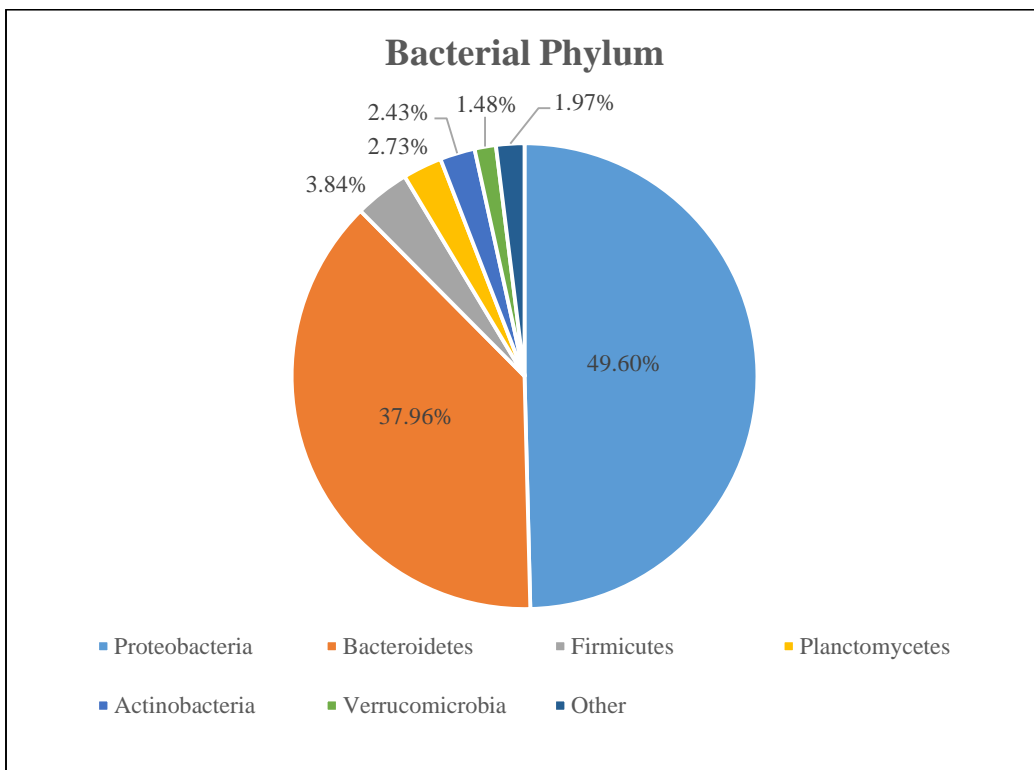


Figure 6-6 Phylum classification of the reactor biomass

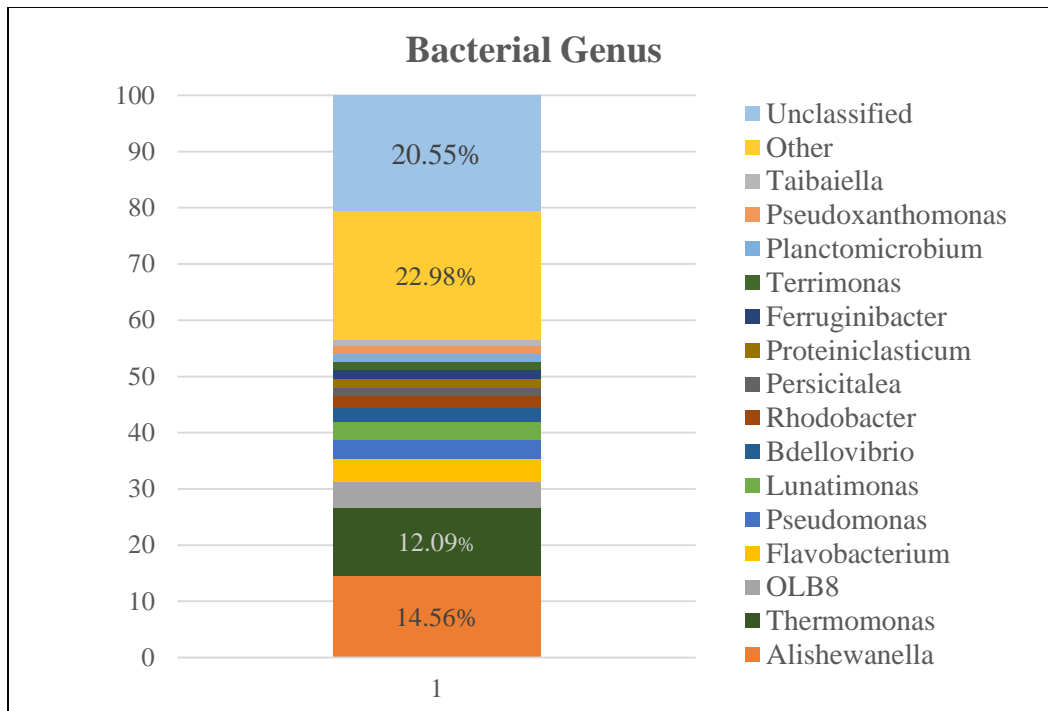


Figure 6-7 Microbial classification of the utilized biomass according to genus

6.4. Conclusion

Several literature and previous research have been carried out to investigate the process of hydrogenotrophic denitrification. However, employing hydrogen as electron donor in denitrification, where nitrite is the electron acceptor instead of nitrate, is a novel process that needs thorough research. This reactor employs hydrogen gas and RAS in reducing nitrite that exists in wastewater, specifically side streams that have high concentration of nutrients which needs advanced treatment and processing to recover resources.

Through a working volume of 1.7 L, the SBR system consisted of 2 cycles per day including 10 min fill/decant in which the reactor is filled with wastewater of 400 mg/L nitrite concentration. The overall performance reached 90% removal efficiency (NRE) corresponding to a loading rate of 400 gN/m³d, and specific denitrification rate (SNDR) of 45 ± 4.5 mgNO₂-N/gVSS/d. The pH of the reactor was within range of 7.5-8.2. The process was in favor of alkalinity production, as proved by literature and previous studies. Microbial strains from the *Proteobacteria* phyla were found to be dominant in the reactor. *Alishwanella* and *Thermomonas* bacteria were the dominant genus in the reactor, which were deduced to be able to utilize nitrite in hydrogen gas abundance. These specific genus were isolated from denitrification system before, which supports the findings.

Chapter 7 Conclusion and Future Work

This chapter gives an overview of the main findings of the thesis project along with recommended future work.

7.1. Conclusion

In this thesis, the main objective was to study the potential of developing an efficient sustainable hydrogenotrophic denitrification system. This approach aims to selectively remove nitrite ions from wastewater with hydrogen gas using autotrophic mixed cultures. The main advantages of such approach are:

- Utilizing a clean energy source such as hydrogen gas make the process better towards the environment and resource management.
- The inorganic carbon source is more available and inexpensive compared to its organic counterparts, as well as the elimination of harmful byproducts produced.

The conducted research was accomplished in two phases: a number of batch experiments and continuous setup. The batch experiments were carried out in small volume (125-250 ml) setup to have a better control and understanding over the parameters, and to identify the most important factors that have higher impact on outcome. Afterwards, the scaling up of the experiment was done in a sequential batch reactor which has a capacity of 2 L, and high loading rate of nitrite. The influent parameters employed in SBR were deduced from the fed batch results. After conducting this multi-stage process of hydrogenotrophic denitrification, the main findings of the conducted research are found to be as follows:

- In the first stage of examining different types of culture as seed for the reactor, the mixed culture, specifically waste activated sludge (WAS) proved to outcompete the other four cultures within the same control parameters. It resulted in a stable nitrite removal efficiency (NRE) of 90% for loading rate of 100 g/m^3 , reaching final concentration of $45 \text{ mgNO}_2\text{-N/L}$. While for the other inoculum cultures, poor performance existed with low removal efficiency that didn't exceed 50% and ranged within 20-30% for most of them.
- The main outcome from the 1st set of experiment was that recycled/waste activated sludge is the proper biomass that can adapt and perform hydrogenotrophic denitrification.
- Followed by that was the second stage, which examined the solids content effect on the nitrite removal rate by selecting different food to mass ratios (F/M), it was concluded that the lower F/M ratio, the higher nitrite removal and growth yield is.
- For F/M: 0.15, and 2000 mg/L bicarbonate concentration; high efficiency of 95%, with nitrite uptake rate of about $3.2 \text{ mgNO}_2\text{/hr}$ was achieved in one of the experiments, leading to effluent concentration less than 5 mg/L . Lower biomass densities with lower solids content, increased the efficiency by 10%.
- The third stage of the experiment involved examining effect of different hydrogen and nitrite concentrations on the performance of hydrogenotrophic denitrification. The lower H/M and N/M ratios of 0.002 and 0.03 respectively, resulted in the best results in regards to nitrite removal efficiency as an overall efficiency of $97\% \pm 2$ was observed in all the samples, especially with alkalinity $2000 \text{ mgCaCO}_3\text{/L}$. Higher H/M and N/M ratio corresponding to 800 mg/L nitrite and 100 mL hydrogen led to a drop by 50% in removal efficiency.

- In the final stage of the research, the SBR setup had overall performance of 90% removal efficiency of nitrite removal corresponding to a loading rate of 400 gN/m³d, and specific denitrification rate (SNDR) of 45 ± 4.5 mgNO₂-N/gVSS/d. The pH inside the reactor stayed within acceptable range of 7.5-8.5.
- Microbial analysis results of SBR indicated strains from the *Thermomonas* and *Alishwanella* genera were abundant in the reactor. These specific genus were isolated from autotrophic denitrification reactors in previous studies.

7.2. Future work and Recommendation

For the upcoming research focus, to optimize the performance of shortcut denitrification, namely hydrogenotrophic denitrification, some parameters and criteria would need further investigation in order to make the system more efficient. The following recommendations are suggested for future research:

- Optimize the main working parameters such as nitrite concentration, pH and alkalinity by the aid of mathematical modeling to better evaluate the results of the reactor, and have a measure of their impact.
- Conduct a kinetic study of the process to investigate kinetic parameters of hydrogenotrophic denitrification, such as growth rate, growth yield of biomass, and decay rate of denitrifying cultures present in hydrogenotrophic conditions. This will lead to a better understanding of growth patterns of mixed culture and the overall process.
- Determine the applicability of coupling the partial nitrification process with hydrogenotrophic denitrification in a single reactor. In this reactor a high nitrogen loading rate would be introduced, in order to reduce ammonia into nitrogen gas directly.

- Improve the feeding protocol of the SBR, so that the fed wastewater can be mixed with the biomass before feeding the reactor to facilitate acclimatization and microbial culture adaptation to environment.
- Further investigation of the microbial cultures found in the reactor, by doing molecular analysis for them such as qPCR, DNA extraction or PCR-DNA amplification, to confirm the strains that are able to reduce nitrite into nitrogen in the presence of hydrogen gas.

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