AMMONIA REMOVAL IN WASTEWATER WITH ANAEROBIC AMMONIUM OXIDATION PROCESS

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ABSTRACT

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Concentrated wastewaters produced in many agricultural and food industries are treated by anaerobic digestion nowadays. However, ammonium ion is not removed in the digestion process, thereby yielding an effluent containing a high concentration of ammonia and low biodegradable COD. This ammonium-rich effluent is usually treated by another wastewater treatment plant, but it is costly and troublesome. Recently, a novel process named Anammox (anaerobic ammonium oxidation) was discovered in which ammonium ion could be converted to nitrogen gas under anoxic conditions with nitrite as the electron acceptor. This innovative Anammox process, as a result, has made the nitrogen removal treatment more sustainable. Current modeling research with the combination of partial nitrification (Sharon) and Anammox reactors has been examined for the removal of ammonia from concentrated wastewater. It was evaluated that about 50% of ammonium ion was oxidized to nitrite in the Sharon process and more than 80% of the ammonia was converted into nitrogen gas in an Anammox reactor. This study aims at demonstrating the feasibility of ammonia removal by providing nitrite directly to the Anammox process without the preceding nitrification step. Results showed that approximately 60% of ammonium removal efficiency and 80% nitrite removal efficiency were achieved in a batch sequencing reactor. Additionally, more than 50% of COD removal was obtained, hence, successfully proving the potential of this method.

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LIST OF SYMBOLS

[ ]	molar concentrations [mol/L]
A	air mass flow rate [kg/h-m^2]
DS	dry solids [kg]
H^+	hydrogen ion concentration [mol/L]
K	Henry's law constant
K_m	substrate concentration when the rate of utilization is half the maximum rate [mass/volume]
k_s	maximum rate of substrate utilization [g/g·time]
K_s	half-velocity constant (i.e. conc. of substrate when $\mu = 0.5\ \mu_{max}$)
k_{T1}	reaction rate constant at temperature T1, °C
k_{T2}	reaction rate constant at temperature T2, °C
$[OH^-]$	concentration of hydroxyl ions [mol/L]
P	product concentration [mg/L]
r	biomass growth rate [organisms/d]
S	concentration of substrate in solution [mg/L]
dS/dt	rate of substrate utilization [g/L·time]
t	time [d]
T	temperature [°C]
W	wastewater mass flow rate [kg/h-m^2]
$x_{n,\,out}$	ammonium ion concentration in wastewater [kg-NH_4^+/kg-water]
X	cell mass concentration [mg/L]

$y_{in, out}$	ammonia concentration in air [kg-NH_3/kg-air]
μ	growth rate constant [d^{-1}]
μ_{max}	maximum specific growth rate [d^{-1}]
μ_{35}	maximum specific growth rate at 35°C [d^{-1}]
θ	temperature correction coefficient

LIST OF ABBREIVATIONS

AFBR	Anaerobic Fluidized Bed Reactor
Anammox	Anaerobic Ammonium Oxidation
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
HPLC	High Pressure Liquid Chromatography
HRT	Hydraulic Retention Time
PCBs	Polychlorinated Biphenyls
PCP	Pentachlorophenol
PVC	Poly Vinyl Chloride
SBR	Sequencing Batch Reactor
Sharon	Single Reactor System For High Rate Ammonium Removal Over Nitrate
TCE	Trichloroethylene
TDO	Total Dissolved Oxygen
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket
UV	Ultraviolet
VSS	Volatile Suspended Solids

CHAPTER 1

INTRODUCTION

In a world full of pollution, contaminated wastewater is considered to be one of the most serious threats to the environment as well as human society. Environmental engineers, with mainly a civil engineering background, have placed a great effort and time on water pollution control (Viessman and Mark, 1998). In the past, the composition of wastewater did not vary greatly since the majority of wastewater originated from domestic sources. Nevertheless, this situation has changed due to the fast growth associated with industrialization. In fact, the municipal wastewater of today is no longer the domestic wastewater of yesterday. Now, municipal wastewater contains more hazardous components and requires more sophisticated treatment systems.

Wastewater contains a variety of contaminants, and nitrogen is considered as one of the undesirable pollutants. Since the 1980s, important experience has been built up regarding biological nitrogen removal at wastewater treatment plants (Peavy *et al.,* 1985). Many wastewater treatment plants have been gradually upgraded and improved their systems in order to accomplish the required nitrogen standard in effluent. However, there are still a number of plants that cannot attain the new requirements regarding total nitrogen removal. The reasons for this can be varied. The major limitation for many treatment plants is a lack of space to apply the conventional nitrogen removal process. Therefore, the wastewater treatment industry is now seeking alternative technologies for nitrogen removal.

In the past, it was believed that the oxidation of ammonia was only restricted to the aerobic environment. Lately, an anaerobic ammonium oxidation process (Anammox) was discovered in a denitrifying pilot plant reactor (Mulder *et al.,* 1995). Consequently, an investigation of different applications of the mentioned process has to be performed for economical and energetical reasons.

1.1 Objective of the thesis

The objective for this proposed research is to study the feasibility and application of an innovative anaerobic treatment process, named Anammox process, for the reduction of total nitrogen in wastewater.

The scope for the proposed research will encompass:

1. Its capability on treating different ammonium and COD concentrations.

2. The possibility of the Anammox application in anaerobic treatment systems by directly adding nitrite as electron acceptor.

3. The design of an anaerobic ammonium oxidation system coupled with the identification of the governing parameters which affect the degradation of ammonium.

CHAPTER 2

LITERATURE REVIEW – STATEMENT OF THE PROBLEM

2.1 Introduction

The literature review has shown that most of the wastewater streams produced in food and agricultural industry are treated by sludge digestion. From the study of Mike and Marc *et al.* (1998), the effluent can contain high ammonia concentrations up to 2000 mg/l. For a long while, this ammonium-rich effluent has been treated by a combination of two processes, aerobic nitrification and anoxic denitrification. Nevertheless, they are not considered as the best option due to the high operational and capital cost. The aforementioned Anammox process was found to be able to convert ammonium ion to nitrogen gas under anoxic conditions by utilizing nitrite as electron acceptor (Mulder *et al.*, 1995). As a result, the review of literature concerning various topics associated with the removal of ammonium ion was carried out in order to accumulate information on the important factors regarding the anaerobic ammonium oxidation process.

The first part of the literature review points out the main sources of ammonia and the major possible conversions for nitrogen removal. Three conversions: nitrification, denitrification and anaerobic ammonium oxidation are discussed since they are significant to aerobic and anaerobic biological N-removal processes. Subsequently, the negative effects of ammonia on human beings and to the aquatic environment are evaluated in order to indicate the necessity of ammonia removal from wastewater streams.

2.2 Sources of ammonia

The principal forms of nitrogen include organic nitrogen, nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3) and ammonium ion (NH_4^+). Ammonia is a colorless gas at ambient temperature and pressure and when ammonia dissolves in water, it forms ammonium cations (NH_4^+) (Ramalho, 1983). Another factor influencing the magnitude of ammonia volatilization is the pH value. NH_3, gaseous ammonia, and NH_4^+, aqueous ammonium ion, are in equilibrium at a pH of 9 approximately (Ramalho, 1983). The equilibrium equations are shown below:

$$\boxed{NH_4^+ \rightleftharpoons NH_3 + H^+}$$

$$\boxed{\frac{[NH_3]\,[H^+]}{[NH_4^+]} = 5.7 \times 10^{-10}}$$

Adapted from world health organization (1986), several physicochemical properties of ammonia are presented in Table 2.1. Figure 2.1 demonstrates the relative concentrations of NH_3 and NH_4^+.

Table 2.1: Physicochemical properties of ammonia

Property	Value
Melting point	- 77.76 °C
Boiling point	- 33.43 °C
Density of vapor	0.6 g/L at 20 °C
Water solubility	421 g/L at 20 °C; 706 g/Lat 0 °C
Vapor pressure	882 kPa at 20 °C

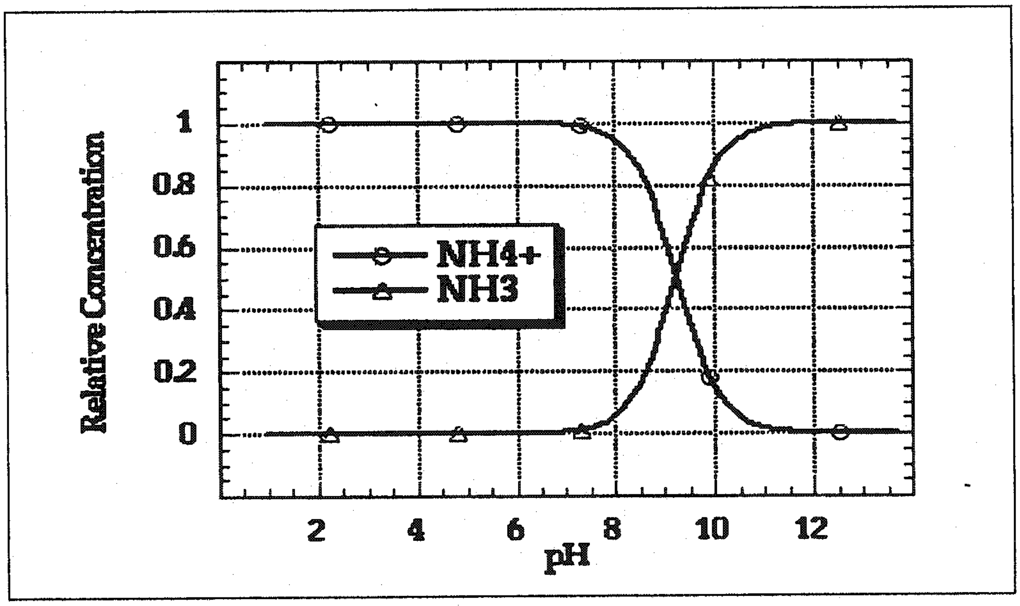


Figure 2.1: Relative NH_3 and NH_4^+ concentrations (World Health Organization Geneva, 1986)

Ammonia that is present in the environment can be attributed to natural processes and through human activities. It is continually released throughout the biosphere by the decomposition of organic waste matter. Therefore, any natural or industrial processes that have nitrogen organic matter available for breakdown represent a potential source of ammonia. Besides, ammonia is one of the most widely used industrial chemicals. It can be utilized as fertilizers, fibres and plastics, explosives, cleaning fluids, scale removing agents, stabilizers and so on (World Health Organization Geneva, 1986). Ammonia and ammonium compounds can also be found in food as leavening agents and composting facilities. Ammonia is released into the aquatic environment from a variety of man-made point sources and from natural and man-made non-point sources.

2.2.1 Point sources of ammonia

Major man-made point sources releasing ammonia into the watercourses include sewage treatment plants, and plants producing fertilizers, steel, petroleum, leather, inorganic chemicals, non-ferrous metals and meat processing plants (Henze *et al.*, 1997).

2.2.2 Non -point sources of ammonia

Non-point sources of ammonia are not easy to be enumerated because they are variable, discontinuous, and differ according to specific land use (Henze *et al.*, 1997). They may be the result of runoff from urban, agricultural, commercial or mined lands. Principal non-point sources of ammonia discharging into the watercourses include fertilizer runoff, animal feedlots, animal wastes spread on the soil, urban runoff, and precipitation. In fact, estimated total runoff quantities and ammonia concentration is extremely complex and no overall estimates are available.

2.3 Microbiological conversions in nitrogen removal

As indicated by Van Loosdrecht and Jetten (1998), nitrogen conversions are always complex due to the large amount of nitrogen compounds involved, and numerous reactions that can occur during biological treatment process. The possible microbial nitrogen conversions are presented in Figure 2.2. This review offers an overview of the possible microbiological nitrogen conversions described in the literature.

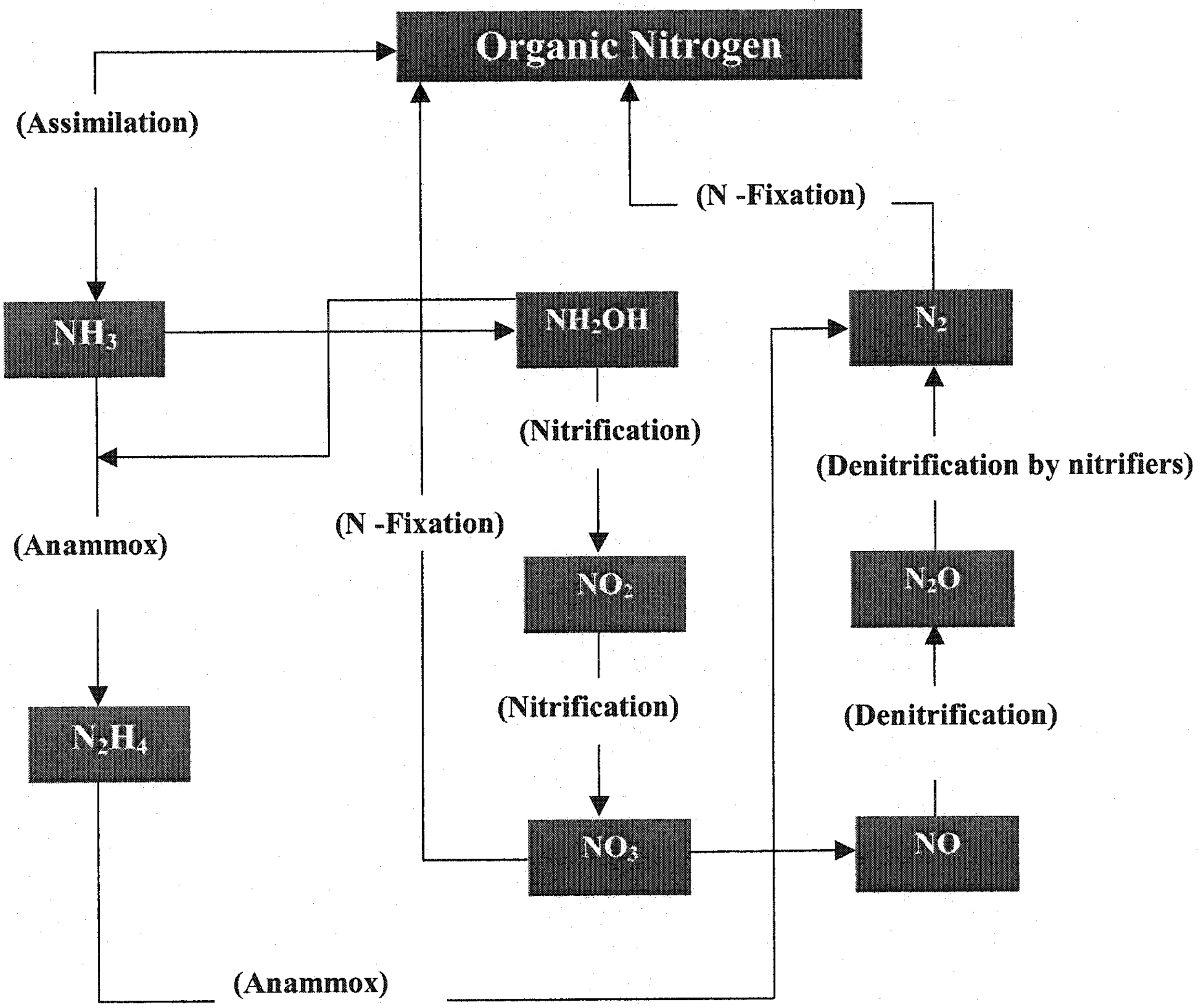


Figure 2.2: Possible microbial nitrogen conversions (Van Loosdrecht and Jetten, 1998)

The goal of nitrogen removal from wastewater is to ensure the concentration of the nitrogen in wastewater effluent is at a desired level. Three major conversions that are considerably crucial to treatment systems include nitrification, denitrification and Anammox. The relevance of these conversions with respect to wastewater treatment processes is discussed in the following section.

2.3.1 Nitrification

Nitrification is generally performed by aerobic autotrophic microorganisms (Reible, 1998). It is a continuous aerobic biochemical action that converts the ammonia/ammonium to nitrite (NO_2^-), and eventually to nitrate (NO_3^-) with molecular oxygen as electron acceptor. To be fully effective, these conversions must have enough oxygen supply. There are many different types of nitrifying bacteria identified. *Nitrosomonas* and *Nitrobacter* are two main nitrifying bacteria that convert the ammonium ion to the nitrite ion, and the nitrite ion to the nitrate ion, respectively (Van Loosdrecht and Jetten, 1998). Nitrification can be achieved in any aerobic process provided that the environmental conditions are satisfied. The activated sludge process is most often used to produce highly nitrified conditions. In the activated sludge systems, the concentrations of ammonium are relatively high whereas the levels of nitrite are very low, so nitrite oxidizers will have to compete for the substrate (Horan, 1990). For this reason, bacteria with a low affinity constant are usually enriched in the laboratory. Other suspended-media (e.g. oxidation ditch) or fixed-media (e.g. trickling filter or rotating biological contractor) processes also can be used to accomplish nitrification.

2.3.2 Denitrification

Denitrification, which is the biochemical conversion of the nitrate ion to nitrogen gas, can be carried out by denitrifying bacteria that utilize the NO_x^- as alternative electron acceptors instead of oxygen (Reible, 1998). To obtain the highest efficiency, denitrification process should be in the absence of oxygen condition. This process also requires an electron donor (COD) which can be provided by organic material or through reduced compounds such as hydrogen or sulphide. The main concern of this conversion is the release of intermediates such as HNO_2, NO, and N_2O from the treatment process into the environment (Van Loosdrecht and Jetten, 1998). Additionally, complete denitrification can only be feasible with the use of methanol, but the costs of purchasing and handling the methanol are considerable.

Similar to the nitrification process, denitrification systems include fixed-media (e.g. anoxic filter, upflow expanded bed granular media and anaerobic fluidized bed) as well as suspended-media (e.g. separate sludge system).

2.3.3 Anammox

Recently, Mulder *et al.* (1995) discovered that ammonium ion was removed in an anaerobic fluidized denitrifying bed. According to Mike *et al.* (1998), this Anammox process encompasses two conversions which are anaerobic oxidation of ammonium to nitrogen gas, and anaerobic oxidation of nitrite to nitrate. The first reaction is a new biochemical pathway to remove ammonium ion from wastewater under anaerobic conditions with nitrite as electron acceptor. The latter reaction is to compensate for the reduction of CO_2 to biomass. This Anammox process can occur in fully autotrophic systems with very long sludge retention times or biofilm systems (Mike *et al.*, 1998). In other words, no organic substrate is required in the nitrogen removal process. Hence, this anaerobic ammonium oxidation (Anammox) provides wide possibilities for future wastewater treatment systems. One point of concern is the extremely low growth rate of these autotrophic organisms - the doubling time is three weeks (Van Loosdrecht and Jetten, 1998). Because of this reason, it is not surprising that these kinds of organisms are not richly found.

Different from the nitrification and denitrification processes, the applications of Anammox in various biological systems are not fully developed. This research, therefore, will mainly concentrate on this conversion process and more details will be discussed in Chapter 3.

2.4 Harmful effects of ammonia

2.4.1 On human beings

In domestic sewage, normally only organic nitrogen and ammonia are present. Nitrate and nitrite usually occur in trace quantities in surface water but may reach high levels due to agricultural runoff, human and animal waste and particular effluents from sewage treatment works. The toxicity of nitrate to humans is mainly attributable to its reduction to nitrite. The major biological effect of nitrite in humans is its involvement in the oxidation of haemoglobin to methaemoglobin, which lacks the ability to transport oxygen to the tissues. This can cause death in children due to oxygen starvation (blue-baby disease) (World Health Organization Geneva, 1986).

2.4.2 On aquatic environment

Proteins and amino acids, which are complex organic matter in wastewater, yield ammonia under bacterial decomposition. The problems associated with ammonia in wastewater include:

- Ammonia is highly soluble in water, and it is lethal to most species of fish at concentrations as low as 0.5 mg/L (World Health Organization Geneva, 1986).
- Nitrification, oxidation of ammonium cation to form nitrate by nitrifying microorganisms, consumes large quantities of oxygen and raises the concentration of nitrate in the watercourse. The following equations describe this process:

$$\boxed{NH_4^+ + 1.5O_2 \longrightarrow NO_2^- + 2H^+ + H_2O}$$

$$\boxed{NO_2^- + 0.5O_2 \longrightarrow NO_3^-}$$

- Nitrogen is known as the main nutrient for living creatures in water, so the increased nitrogen loads can stimulate the growth of the algae. Algae have proven to be ecologically harmful to the other inhabitants of the water not only because it increases the pH values of the water during the day, but also because it takes up oxygen and gives off carbon dioxide at night.

- Ammonia in waters used for water supply increases the use of chlorine to achieve a free chlorine residual in disinfection.

2.5 Conclusion

Nitrogen removal is an important aspect of today's wastewater treatment processes. A literature review demonstrated that ammonium removal by activated sludge system is complex because of the large amount of nitrogen compounds involved and the numerous reactions that can occur.

Anaerobic digestion is a common way to treat concentrated wastewaters and sludges. Nevertheless, ammonium is not removed in the digestion process, thereby yielding an ammonium rich effluent and low in biodegradable COD. Treatment of this ammonium rich effluent is usually troublesome and expensive. The novel anaerobic ammonium oxidation process provides another low cost alternative to conventional treatment plant for nitrogen removal.

The literature review did not demonstrate the direct application of Anammox process in an anaerobic treatment plant. In addition, the impact of Anammox process on the anaerobic systems was not shown. As a result, it is indispensable to identify and classify the main factors affecting the ammonium removal in anaerobic treatment systems.

CHAPTER 3

COMPARISON OF DIFFERENT TECHNOLOGIES FOR AMMONIA REMOVAL

3.1 Introduction

Ammonia removal can be accomplished by physical, chemical or biological processes. In general, biological processes provide the most economical means for nitrogen removal in wastewater effluents and they have numerous advantages over physical-chemical processes.

- Lower capital costs, as well as operations and maintenance costs.
- Less energy requirements. This is particularly important in anaerobic processes where the addition of oxygen for aeration is not required and the production of methane gas can be an energy source.
- Larger range of applications with the exception of a few persistent or recalcitrant contaminants (e.g. PCBs and highly chlorinated solvents).

Even though the removal of ammonia is more commonly achieved by means of biological reaction, land space is a main limiting factor. Also, if ammonia concentrations are too high, such high removal rates may not be practical. This chapter includes classification and description of various processes for ammonia removal from wastewater.

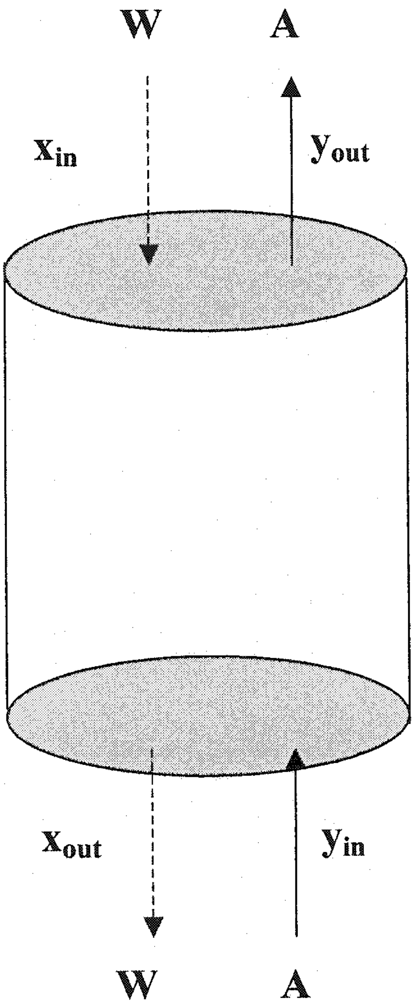
3.2 Physical process for ammonia removal

3.2.1 Air stripping

Air stripping of ammonia involves the passage of large quantities of air over the wastewater, thus causing the partial pressure of the ammonia gas within the wastewater to convert the ammonia from the liquid phase to the gas phase (Peavy *et al.*, 1985). Ammonia nitrogen is present in wastewater in two forms. The first is dissociated ammonia, NH_4^+, also referred to as ammonium ion. The second is undissociated ammonia, NH_3, known as ammonia gas.

$$\boxed{NH_4^+ + OH^- \rightleftharpoons NH_3 + H_2O}$$

Increase of the pH shifts the equilibrium more to the right, thus causing the major portion of the ammonia to be in the form of ammonia gas molecules. According to Reynolds and Richards (1996), for the complete conversion of ammonium ion to the gas phase (NH_3), the pH must be about 11 or greater. As the pH value is well above the normal pH in wastewater, pH adjustment by using lime is usually required prior to air stripping. A countercurrent spray tower is found to be the most efficient reactor for air stripping. The tower basically is composed of a fan to draw air through the tower, a packing to give intimate air and water contact and a distribution system (Reynolds and Richards, 1996). Figure 3.1 shows a schematic diagram of countercurrent for ammonia stripping tower.



W = Wastewater mass flow rate

A = Air mass flow rate

x = Ammonium ion concentration in wastewater

y = Ammonia concentration in air

Figure 3.1: A schematic diagram of an air stripping tower

Air stripping can be considered as one of the most economical processes for ammonium ion removal in wastewater treatment. Yet, there are some serious limitations to this process and adjustment of pH value is one of the significant disadvantages in air stripping. The other commonly encountered difficulty in this process is the temperature. Air stripping tower cannot operate in subfreezing weather due to icing. As a result, temperature control is necessary, but it can raise the operating cost. Noise and air pollution are also the associated problems with the air stripping method.

3.3 Chemical processes for ammonia removal

3.3.1 Ion exchange

The ion exchange process includes a chemical reaction between ions in a liquid phase and ions in a solid phase. Cation exchange utilizing clinoptilolite, which is a natural zeolite, for ammonia removal has been introduced since 1970. In the process, the wastewater is first treated by multimedia filtration, then by carbon filtration, and eventually by ion exchange using a fixed-bed column containing clinoptilolite (Gaspard *et al.*, 1983). The usefulness of clinoptilolite is mainly attributed to its selective ability for the removal of ammonium ions in the presence of divalent metallic ions (Celik *et al.*, 2001). From the study of Gaspard *et al.* (1983), regeneration of clinoptilolite should be done with a brine solution of about 2% NaCl, which is regenerated by electrolysis.

By utilizing clinoptilolite to remove ammonia in wastewater, the efficiency can be achieved from 94% to 97% in comparison with 90% removal by air stripping (Celik *et al.*, 2001). However, the cost of ion exchange is more expensive than air stripping. The application of ion exchange for ammonia reduction in wastewater should be considered when it is necessary to have complete removal of ammonium ions at concentrations about 70 mg-N/L (Peavy *et al.,* 1985).

3.3.2 Breakpoint chlorination

Chlorination by chlorine gas will oxidize ammonia to produce intermediate chloramines, and, eventually, to form nitrogen gas and hydrochloric acid (Horan, 1990). The reaction that oxidizes the ammonia in chlorination process is:

$$\boxed{2NH_4^+ + 3Cl_2 \longrightarrow N_2 + 6HCl + 2H^+}$$

Studies have indicated that the amount of chlorine required is independent of temperature from 4.4°C to 37.8°C, and approximately 95% to 99% of the ammonia will be oxidized to nitrogen gas (Reynolds and Richards, 1996). Some undesirable side reactions may occur to form dichloramine, trichloramine, which is very unbearable in odor, and nitrate ions. Nevertheless, if the pH is maintained within the range 7 to 8, these side reactions can be minimized (Reynolds and Richards, 1996). If the alkalinity is not sufficient enough to keep this pH range, a base like NaOH has to be added for neutralizing purposes. The disadvantages of using chlorination comprise of the increasing dissolved solids in wastewater and high cost of operation. Furthermore, according to the World Health Organization (1986), the application of chlorination for ammonia removal may be phased out in the future due to the adverse health effects of trihalomethanes that are formed in chlorination.

Both breakpoint chlorination and ion exchange involve complex technologies that are high cost and require skilled operators. Ammonia stripping has the commonly perceived significant disadvantage of requiring pH adjustment. The advantages and disadvantages of air stripping, breakpoint chlorination and ion exchange are summarized in Table 3.1.

Table 3.1: Comparison of technologies available for the physical and chemical removal of ammonia from wastewater

Process	Advantages	Disadvantages
Air Stripping	✓ Easy to control to achieve required removal rates ✓ Not sensitive to toxic substances ✓ Process can meet total nitrogen standards ✓ Less operational cost in comparison with other chemical processes	⌘ It is temperature sensitive and requires temperature control ⌘ May have varied performance subject to ambient temperatures ⌘ Requires pH adjustment ⌘ Carbonate scaling of packing
Breakpoint Chlorination	✓ 99% of ammonia removal can be achievable ✓ Not affected by toxic components ✓ Not effected by temperature ✓ Low capital cost ✓ Can be used where land space is limited	⌘ High operating cost ⌘ Produces high chlorine residuals which are toxic to aquatic environment ⌘ Requires pH control ⌘ Chlorine addition raises total dissolved solids
Ion Exchange	✓ Produces a reclaimable aqueous ammonia product ✓ May be able to meet total nitrogen standards ✓ Easy to control the product quality	⌘ Affected by other cations if they are in high concentration ⌘ High capital and operating costs ⌘ Requires better control and highly skilled operator

3.4 Biological processes for ammonia removal

Biological treatment processes are widely used for the degradation of organic contaminants derived from residential, agricultural and industrial activity. The use of microorganisms for biotransformation or complete mineralization is readily seen in the environment.

Most biodegradable organic contaminants can be decomposed by either aerobic or anaerobic processes at about the same rates per unit of biomass. In the past, anaerobic treatment was not widely used due to a basic misunderstanding of its capabilities. Today, however, with the discovery of the inherent disadvantages of aerobic process coupled with the recognition of the superior abilities of anaerobic treatment, engineers are placing more effort on developing anaerobic treatment for wastewater.

3.4.1 Aerobic and anaerobic treatment

In both treatments, aerobic as well as anaerobic, the primary principle is organic pollutants act as the energy source (electron donor) and oxygen, nitrite, nitrate, sulfate, or carbon dioxide serves as the electron acceptor. When organic contaminants are degraded by microorganisms, those pollutants will lose electrons in the reaction. Based on the principal of conversation of mass, there must be an electron acceptor to receive the losing electron. The main difference between aerobic and anaerobic treatments is that aerobic processes make use of oxygen as the electron acceptor but anaerobic processes utilize sulfate or carbon dioxide or other components as an electron acceptor.

In fact, both aerobic and anaerobic processes are conversion processes that consist of biotransformation and mineralization.

- Biotransformation is a process to transform the complex wastes to simpler form.
- Mineralization is a process to convert the organic contaminants into carbon dioxide and water as well as the excess biomass - activated sludge.

Eventually, the produced gas is collected and some of the activated sludge can be reused for the treatment processes. Nevertheless, the majority of activated sludge is practically non-biodegradable and it causes a giant disposal problem. Hence, minimizing biomass synthesis is one of the greatest concerns in biological wastewater treatment.

3.4.2 Advantages and disadvantages of aerobic biological treatment

Under aerobic conditions, the microbes transform some of the carbon pollutants into carbon dioxide (CO_2) and the emission of this gas causes greenhouse gas problems. Moreover, using oxygen as an electron acceptor, the microorganisms in aerobic processes gain more free energy. In other words, greater amounts of excess sludge are produced. As a result, not only is the cost of disposal increased relatively, but it also results in a large negative impact on the environment. Furthermore, the food and the microorganism utilization rate of aerobic processes is less than twice the normal rate for anaerobic processes. For that reason, the aerobic volumetric loading rates are usually 10 times lower than for the anaerobic process (Henze *et al.*, 1997).

Under some conditions, aerobic treatment may still be the better choice for water quality treatment because of its rapid startup, low ammonium ion (NH_4^+) concentrations and ability of nitrification. However, increasingly restrictive controls on air emission, hazardous waste and activated sludge disposal as well as groundwater contamination have encouraged many environmental engineers to search for other alternative treatment process.

3.4.3 Pros and cons of anaerobic biological treatment

In contrast to aerobic treatment, anaerobic biomass converts the organic contaminants to carbon dioxide (CO_2) and methane gas (CH_4). The advantage of the production of methane gas is owing to its fuel value. Moreover, the total capital costs and power consumption are reduced because aeration equipment is not required and less activated sludge is produced. In addition to the reduction of handling costs, less excess sludge results which minimizes environmental impacts.

Two main concerns in anaerobic treatment are long start up time requirements to achieve design biomass inventory levels and the inability to nitrify ammonium. Also, higher temperature and detention times for the growth of anaerobic microorganisms are required which is disadvantageous. Nevertheless, due to the extensive research by environmental engineers who have been improving the anaerobic technology, these disadvantages can be minimized or even avoided today with proper operation and monitoring. The advantages and disadvantages of each of the biological treatments are summarized in Table 3.2.

Table 3.2: Comparison of aerobic and anaerobic biotechnology treatments

	Advantages	**Disadvantages**
Aerobic Biotechnology Treatment	✓ Fast start up for the growth of microbes inventory ✓ Nitrification is possible ✓ Low ammonium (NH_4^+) requirement for the reaction ✓ Has been used extensively; familiarization by most environmental engineers	✘ Production of carbon dioxide only ✘ Low volumetric organic loading rates ✘ High biomass synthesis rates ✘ High nutrient requirements ✘ Aeration energy required, increase in capital costs and power consumption ✘ High operational and waste biomass disposal costs
Anaerobic Biotechnology Treatment	✓ Production of methane gas and elimination of off-gas air pollution ✓ Higher influent loading rates ✓ Fewer excess sludge formation, decrease total costs and negative effects to the environment ✓ No aeration energy need ✓ Anaerobic biomass is still active for months or years ✓ Conservation of energy that is beneficial to ecology and economy	✘ Long start up for the development of microbes inventory ✘ Nitrification is difficult ✘ High ammonium ion (NH_4^+) concentrations required ✘ Higher temperature is indispensable, usually around 35°C ✘ Higher detention time

3.4.4 Conventional biological N-removal system

The conventional nitrogen removal system consists principally of the two processes: nitrification and denitrification. These two processes will biologically convert ammonia to nitrogen gas. Nitrification can be achieved in an activated sludge process provided that the conditions in the plant are appropriate for maintaining a large population of nitrifying bacteria in the biomass. Viessman and Mark (1998) indicated that the theoretical reaction of the oxidation of ammonia to nitrate requires 4.19 to 4.57 mg of oxygen for every mg of ammonia oxidized. Dissolved oxygen should be kept between 2 and 4 mg/L in order not to inhibit the growth of the nitrifying bacteria. The optimum pH and temperature are 7 and 25°C, respectively (Viessman and Mark, 1998).

For denitrification to occur, a good nitrification process must first take place. As mentioned in previous chapter, denitrification is under anoxic conditions and no free oxygen is available. However, the denitrifying bacteria require a carbon source for their growth. So extra sources of carbon such as raw wastewater or methanol are sometimes provided to the anoxic reactor so as to promote the rate of the biomass growth. The optimum pH for denitrification is 7.0 to 7.5 (Henze *et al.,* 1997). The nitrification – denitrification flow diagram is presented in Figure 3.2.

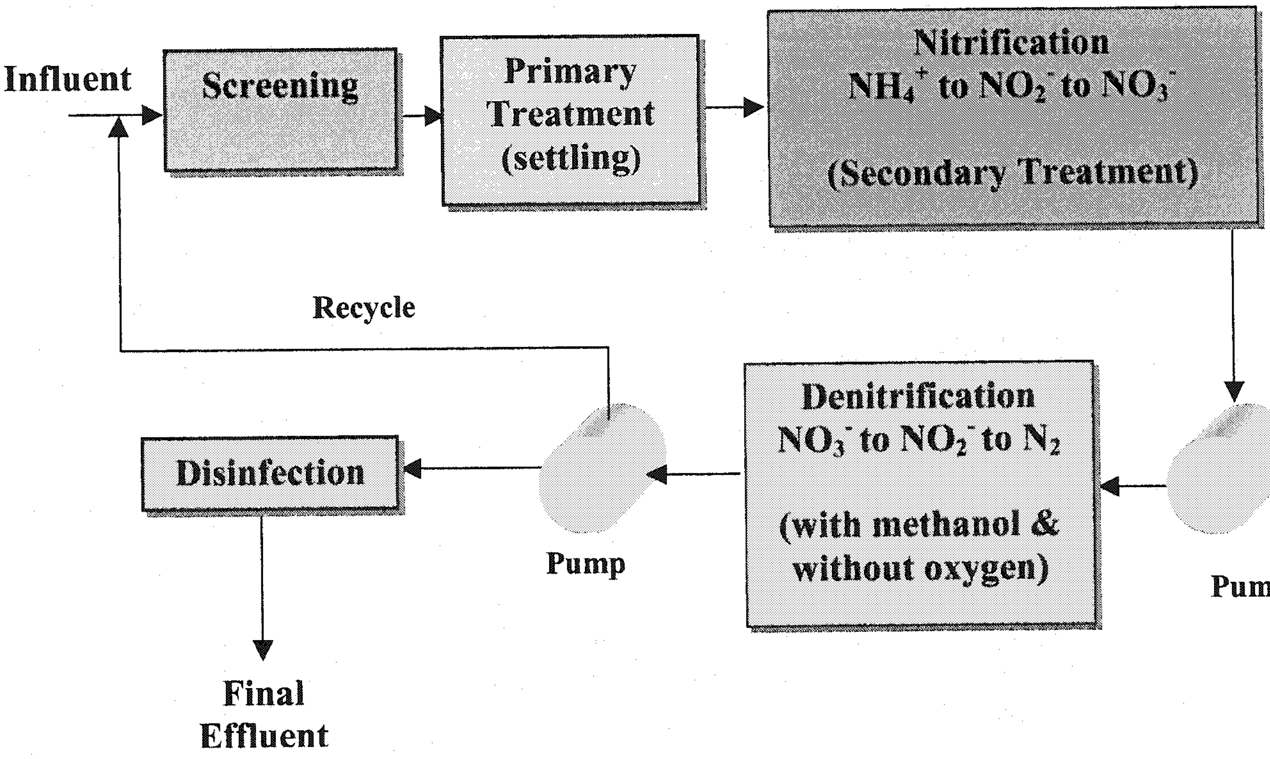


Figure 3.2: Basic schematic flow of nitrification – denitrification process

Nevertheless, N-removal by nitrification and denitrification cannot objectively be considered as sustainable processes. The reasons include:

- Nitrification requires a lot of energy for aeration.
- Large nitrification volume is necessary because of the low growth rate of nitrifiers.
- Denitrification requires sufficient organic carbon. If the COD in the wastewater is not sufficient, an additional carbon source (e.g. methanol) is indispensable.
- Nitrification and denitrification are both sources of atmospheric N_2O.

3.4.5 Principle of anaerobic process

Anaerobic treatment can be utilized for wastewater as well as for digestion of sludge. It has become more and more popular due to its advantages over aerobic processes. On the other hand, the start up and control of these processes are relatively complex because of the low growth rate of their microorganisms and their high degree of sensitivity to environmental conditions. Because of its complexity, the elementary theory of anaerobic treatment and some of the anaerobic systems are studied in this section.

The description of all the chemical activities performed by a cell is defined as metabolism. According to Speece (1996), anaerobic biodegradation comprises four stages and this complicated process is shown in Figure 3.3. The complicated organic compounds in the wastewater are first hydrolyzed to simple organic constituents, and then these simpler organics are converted mainly to long chain organic acids by the acidogens. Up to this stage, there is no COD reduction since the process is simply the conversion of complicated organic molecules to long chain fatty acids. Subsequently, the volatile acids longer than two carbons are broken down to acetate, hydrogen gas and carbon dioxide. Finally, the acetate and the carbon dioxide are converted to methane gas by methanogens in the presence of hydrogen gas.

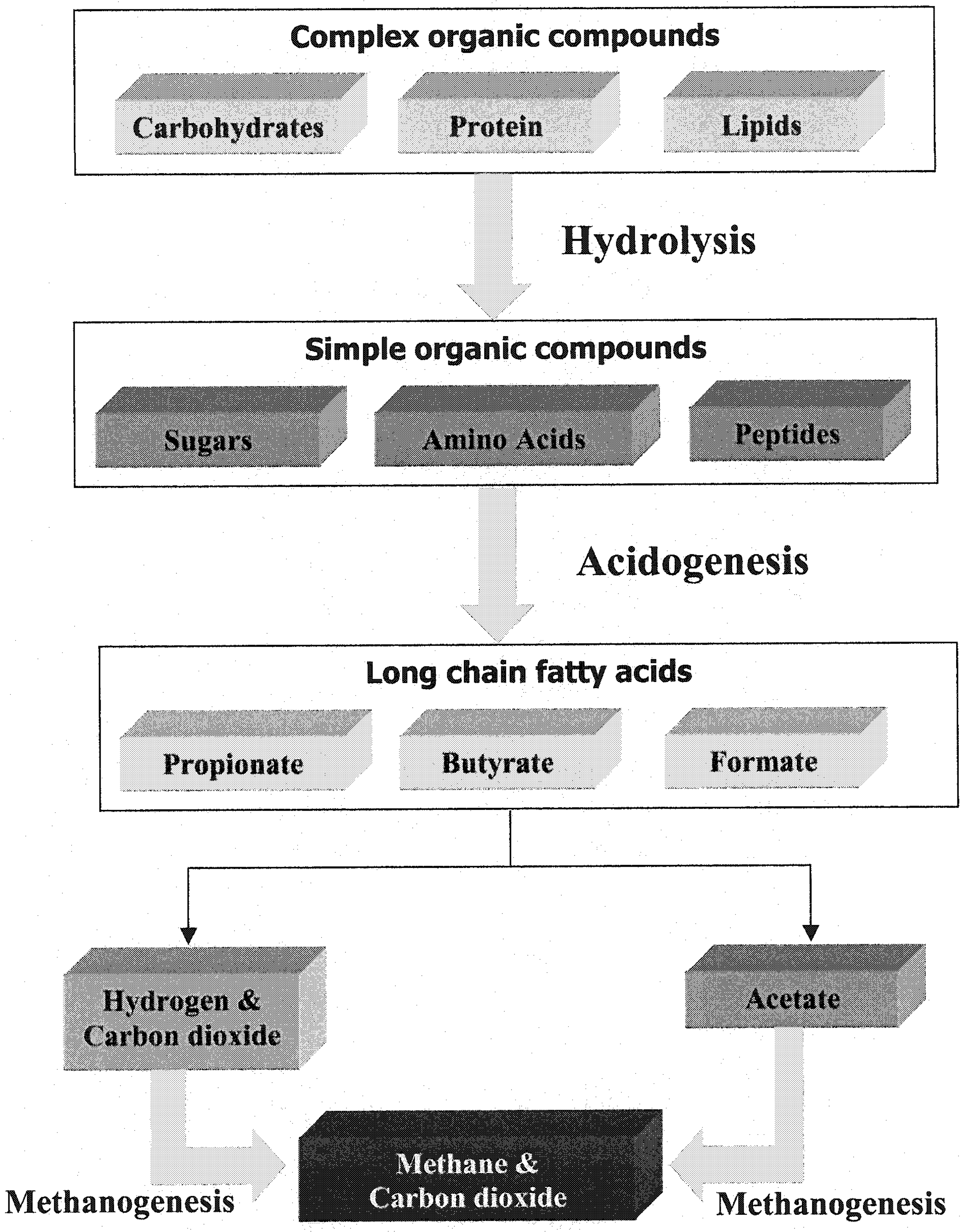


Figure 3.3: Metabolic steps resulting in methanogenesis (Speece, 1996)

Anaerobic systems can be subdivided into two distinct groups, namely low rate and high rate. For the low rate system, the retention time is so long that it leads to large installations. For this reason, the modern high rate anaerobic systems are becoming an increasingly attractive anaerobic system for the treatment of wastewater. High rate anaerobic reactors are developed in order to shorten the retention time and thus decrease the volume of the reactors.

The first start up is the most critical factor in anaerobic process since it requires a long period of time and may cause the failure of the process. The aim of the start up with respect to high rate anaerobic systems is to cultivate and to maintain a high concentration of active biomass in the reactor. The high rate reactors that are commonly used included the upflow anaerobic sludge blanket (UASB) reactor, the fluidized bed reactor and the multiplate reactor.

3.4.5.1 Upflow anaerobic sludge blanket (UASB)

In many countries, the UASB reactor is widely applied in the field of wastewater treatment due to its high efficiency and good settling ability. The UASB reactor consists of two parts, which are a reaction zone, in the lower part, and a separation zone, in the upper part (Boardman and McVeigh, 1997). From the study Boardman and McVeigh (1997), they indicated that the wastewater flows upward through a reaction zone containing suspended granules of biomass where the organic matter is degraded. The organic matter is converted to methane and carbon dioxide under anaerobic conditions and gas bubbles are formed. In addition to the formation of gas, granules that carry a high concentration of biomass are produced. These gas bubbles raise some of the granules in the reactor, but most of them will settle in a lower zone as their settling velocity is greater than their upflow velocity. Boardman and McVeigh (1997) also explained that the mixture of gas, liquid and biomass are then separated in the separation zone. The separation of gas and solids is significant since a large amount of biomass loss can reduce the performance of the reactor dramatically. Therefore, a special gas-liquid-solids separator is utilized in the reactor.

UASB reactors have numerous disadvantages that are difficult to overcome or increase the cost of the treatment system. Sensitivity to pH, temperature and toxic compounds, creation of dead zones, long start-up times and production of odorous compounds have been documented (Seghezzo *et al.,* 1998). In addition, UASB reactors are not suitable for low strength wastewaters (COD < 500 mg/L) (Mulligan, 2002). Although chemical addition (buffer) may be necessary for industrial effluent treatment, it is typically not needed for domestic wastewater and sewage. The bacteria adapt well to low temperatures and can tolerate toxic inputs much better than anaerobic reactors. Despite the disadvantages mentioned above, UASB reactors have been used to degrade pentachlorophenol (PCP) with up to 99% efficiency (Lettinga *et al.,* 1980), crab processing wastewater with 91% efficiency (Boardman and McVeigh, 1997) and for nitroaromatic compounds (Donlon *et al.,* 1996). Typical design parameters for UASB reactors are shown in Table 3.3.

Table 3.3: Design parameters for UASB reactors (Mulligan, 2002)

Design Parameter	Value
Organic Loading Rate	0.5 – 30 kg/m$^3\cdot$d (higher than aerobic systems)
VSS : COD	< 1
Treatable COD Concentration	500 – 20 000 mg/L
Hydraulic Retention Time	4 to 8 h
Temperature Range	15 to 28°C
Ammonia Nitrogen Load	2.4 – 3.4 kg NH_4^+-N/m$^3\cdot$d

3.4.5.2 Anaerobic fluidized bed reactor (AFBR)

The AFBR is one of the most promising and recently used anaerobic technologies. In an AFRB, wastewater flows upward through a bed of suspended media to which the bacteria are attached. Figure 3.4 illustrates the anaerobic fluidized reactor configuration. The support media consists of small inert particles such as sand, high-density plastic beads, granular activated carbon, PVC particles, and other artificial materials (Collins *et al.,* 1998). The bed is fluidized and can be expanded by the high upflow velocity, thus having more particle movement. The velocity must not be so high that the biomass is swept away from the reactor entirely (Rintala, 1991). Because of the increased movement and friction between particles, biofilm formation is minimized. The intense expansion also increases the mass transfer of substrates and stimulates the biological growth. A number of disadvantages of this type of reactor include the longer period of time for biofilm formation, the high pumping costs and sophisticated operational control.

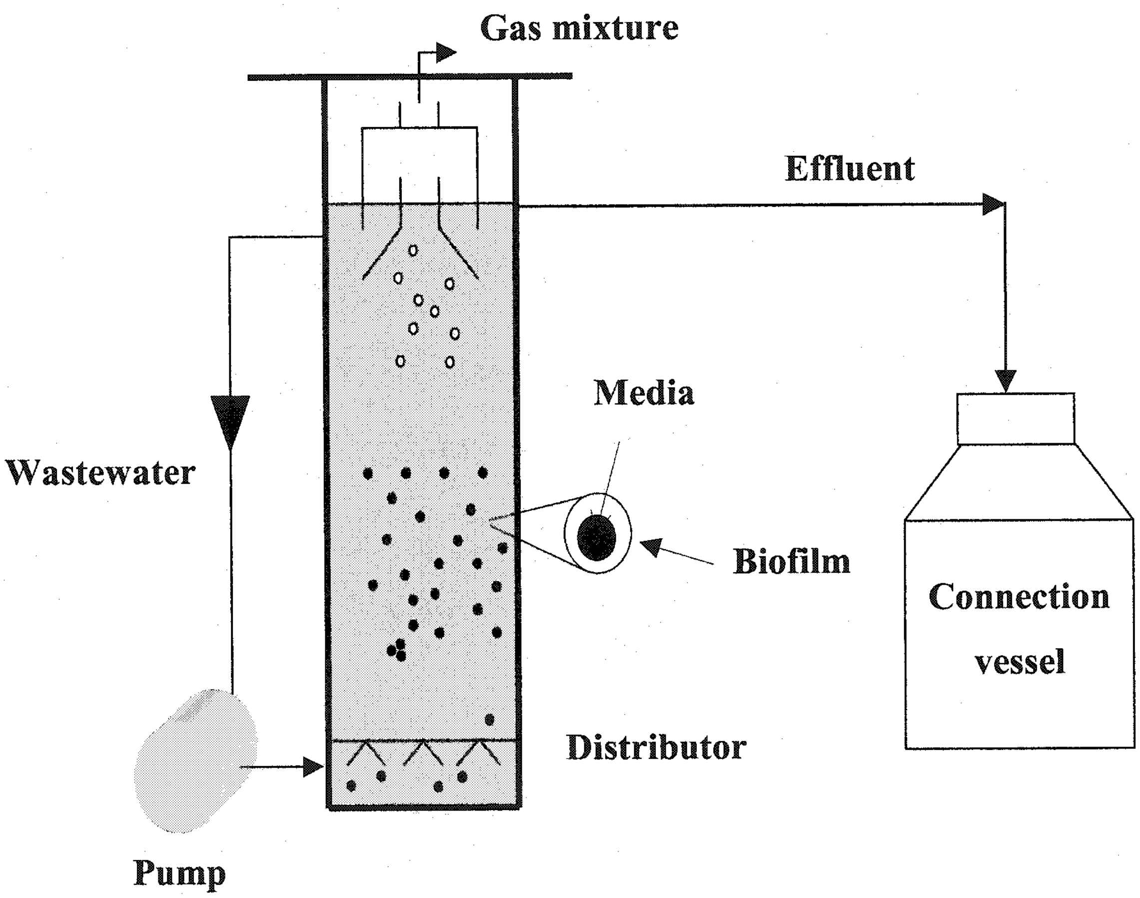


Figure 3.4: Schematic representation of the anaerobic fluidized bed reactor (AFBR)

3.4.5.3 Multiplate reactor

The multiplate reactor technology was developed by SNC-Lavalin in Montreal, Quebec, Canada. It has been used for a decade and this modern technology can be utilized to treat wastewater containing a high concentration of organic matter, especially from industrial and agricultural sources. The multiplate reactor is vertical and it is composed of outer shell, plates, parallel feed entrances and lateral gas exits (Mulligan, 2002). The function of the plates is to permit the upward flow of the wastewater as well as the gases produced during the treatment process. The plates in the reactor also prevent the biomass from falling down to the base of reactor thereby allowing for the anaerobic reaction to proceed over several sludge beds. Biomass granules are formed in each compartment through the degradation of an easily degradable organic compound. The influent enters the reactor via one of the parallel entrances, and Figure 3.5 depicts this system in detail. The gases formed in the reactor (methane and carbon dioxide) ascend and hit the plates. A portion of the gas continues to ascend into the adjoining compartment, while the remaining gas leaves the reactor by a gas exit situated under the same plate. The gas that passes through the plate continuously cleans passage points and prevents the formation of dead zones and short-circuiting. Total COD removal rates of greater that 93% and soluble COD removal rate of 98% have been achieved (Mulligan, 2002).

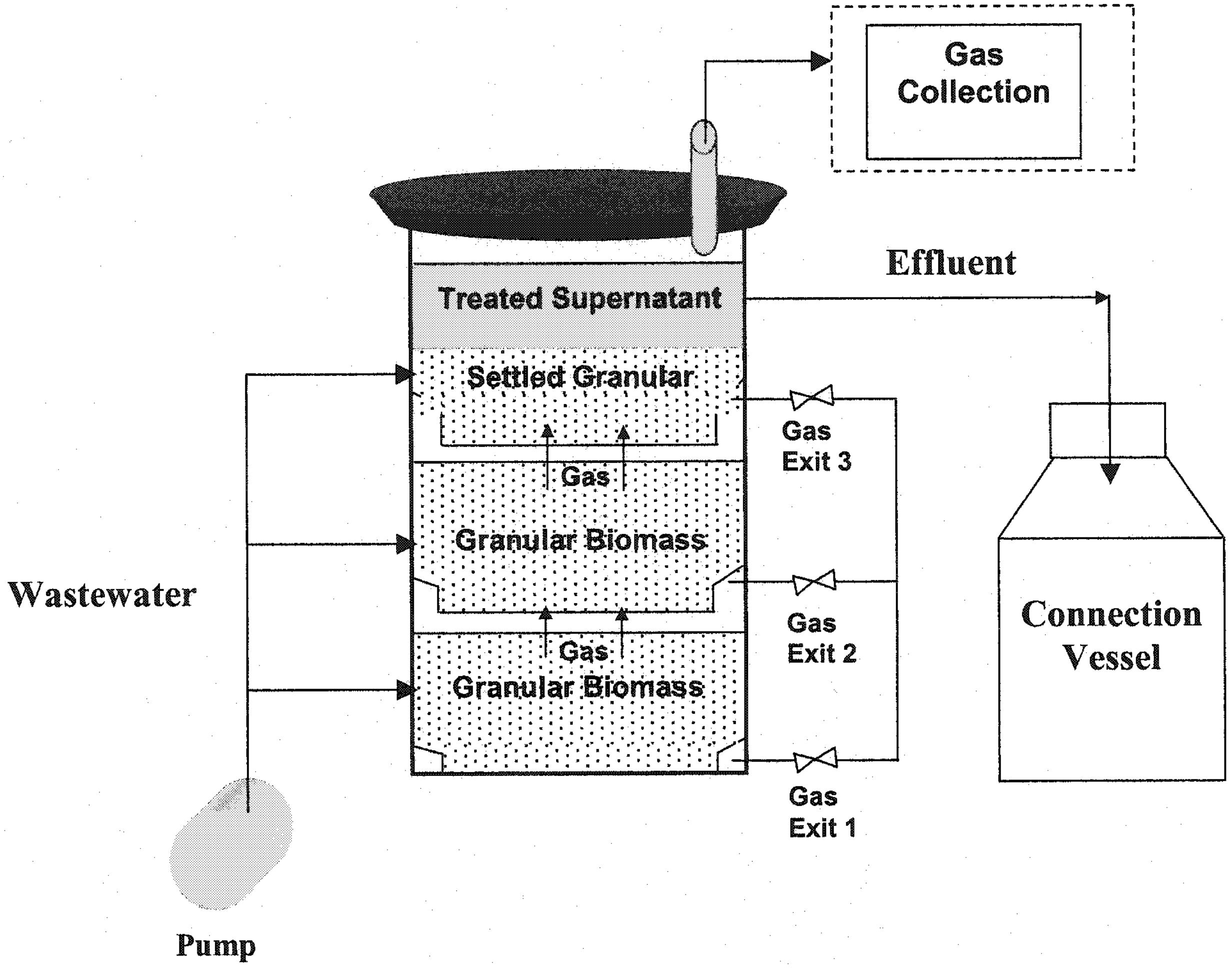


Figure 3.5: Schematic representation of the multiplate reactor

3.4.5.4 Sharon-Anammox reactor

The combination of the partial nitrification process (Sharon) and the Anammox process has been tested successfully using sludge digester effluent (Mike *et al.*, 1998). Van Dongen *et al.*, (2001) also proved that this innovative biotechnological combination improved significantly the treatment of ammonium rich wastewater with minimal energy use. Figure 3.6 demonstrates a fundamental flow scheme of the Sharon-Anammox reactor.

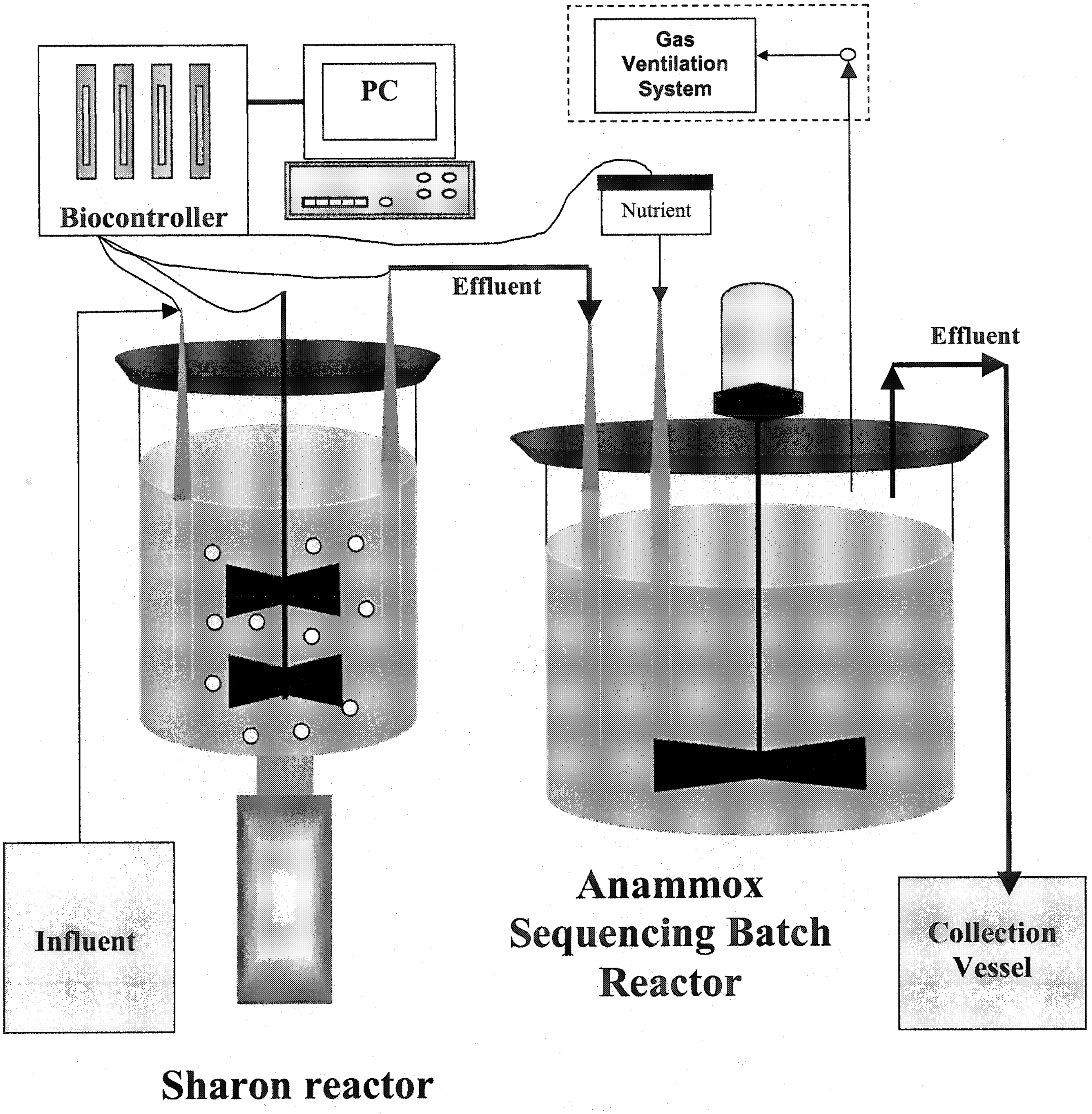


Figure 3.6: Schematic representation of the Annamox sequencing batch reactor using effluent from the Sharon reactor as feed stuff

Sharon, also known as a partial nitrification process to remove nitrogen from waste streams with a high ammonium concentration, is a single reactor system for high rate ammonium removal over nitrate. The Sharon process can be operated in a single, stirred tank without any biomass retention. In other words, the sludge age is equal to the hydraulic detention time (Mike *et al.,* 1998). According to Van Dongen *et al.* (2001), the process is performed at temperature above 25°C using fast growing ammonium oxidizers to treat the ammonium rich influent. In Sharon process, ammonium ion is converted to nitrite under aerobic conditions by ammonium-oxidizing bacteria and it can approximately produce a 50:50 mixture of ammonium and nitrite in the system (Van Dongen *et al.,* 2001). The following equation describes the process in the Sharon reactor:

$$\boxed{NH_4^+ + 1.5\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+}$$

The test results are tabulated in Table 3.4. The effluent of the Sharon reactor was then used as the influent of the Anammox sequencing batch reactor.

Table 3.4: Conversion of NH_4^+ in the Sharon reactor during the test period (Van Dongen *et al.*, 2001)

PARAMETER	UNIT	STEADY STATE OPERATION	TOTAL PERIOD (240 days)
Influent $NH_4^+ - N$	kg/m^3	1.18 ± 0.14	1.17 ± 0.25
Effluent $NH_4^+ - N$	kg/m^3	0.55 ± 0.10	0.6 ± 0.2
Effluent $NO_2^- - N$	kg/m^3	0.6 ± 0.1	0.55 ± 0.2
Effluent $NH_3 - N$	kg/m^3	0	0
pH	kg/m^3	6.7	6.8
$NH_4^+ - N$ conversion	%	**53**	**49**
N- conversion	kg/m^3	0.63 ± 0.1	0.52 ± 0.2

Anammox sequencing batch reactor (SBR) is a newly developed anaerobic biological treatment system for removing ammonium ion and nitrite efficiently. The conversion of ammonium ion takes place without the presence of an organic carbon source and under anoxic conditions. The bacteria utilize the available nitrite as an electron acceptor to convert ammonium ion into nitrogen gas. The following equation illustrates the Anammox reaction (Van Dongen *et al.*, 2001):

$$\boxed{NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \longrightarrow 1.02\,N_2 + 0.26\,NO_3^- + 2.03\,H_2O + 0.066CH_2O_{0.5}N_{0.15}}$$

A clear advantage of the Anammox SBR is to have low sludge production. Nevertheless, in order to keep all the Anammox biomass in the reactor, it is necessary to have an efficient biomass retention system (Van Dongen *et al.*, 2001). In addition, long start-up times will be required to grow enough biomass. The test results of the Anammox SBR are given in Table 3.5.

Table 3.5: Ammonium ion and nitrite removal in Anammox SBR (Van Dongen *et al.*, 2001)

PARAMETER	UNIT	STEADY STATE OPERATION
Test period	day	155 - 190
Influent $NH_4^+ - N$	kg/m^3	0.45 ± 0.02
Effluent $NH_4^+ - N$	kg/m^3	0.068 ± 0.02
Influent $NO_2^- - N$	kg/m^3	0.46 ± 0.03
Effluent $NO_2^- - N$	kg/m^3	1.25×10^{-4}
$NH_4^+ - N$ removal	%	**85**
$NO_2^- - N$	%	100
Total N removal at nitrite limitation	(kg $N_{tot}/m^3_{reactor}$/day)	0.96 ($\pm$ 0.07)
Average specific N_{tot} removal at nitrite limitation	(kg N_{tot}/kg DS/day)	0.70($\pm$ 0.07)

In the Sharon process, approximately 50% of the ammonium ion from sludge water can be oxidized to nitrite. Ammonium ion and nitrite from the effluent of the Sharon reactor can be converted in an Anammox reactor to nitrogen gas. The Anammox reactor can be started up using activated sludge. The start up period lasts usually a few months. After the Anammox process is started up, it can be operated for a long period.

From this case study, it shows that by the use of the combined Sharon-Anammox process, the nitrogen removal will no longer require the input of COD, thus this system can be operated independently. This research also demonstrates that in the nitrite limited Anammox reactor, all nitrite is removed and the surplus ammonium remained. Given the positive cost saving as well as substantial reduction in the energy, a full scale implementation can be expected in the near future.

CHAPTER 4

MODELING OF FACTORS INFLUENCING THE ANAEROBIC AMMONIUM OXIDATION PROCESS

All biological treatment systems have to control or manipulate the growth of microorganisms, so it is important to understand some of common requirements of microbial growth. In addition, there are a number of factors that can influence the biological processes. Therefore, this chapter includes classifications and descriptions of microorganisms as well as identifications of different factors in biological treatment.

4.1 Microorganisms

Microorganisms play an important role in the treatment of wastewater containing decomposable residues. Typically, microorganisms are subdivided according to cell type. Based on the cell structure, microorganisms can be divided into two classes: Prokaryotes and Eukaryotes.

- Prokaryotes are simple and one-cell structure. They are small (< 20 microns) with a primitive nuclear area containing one chromosome (Peavy *et al.,* 1985).
- Eukaryotes are larger cells (> 20 microns) with a more sophisticated cell structure (Peavy *et al.,* 1985). Each cell consists of distinct membrane-bound nucleus with many chromosomes. They may be either single-celled or multicellular, and a complex life cycle can be found (Peavy *et al.,* 1985).

As summarized in Figure 4.1, prokaryotic cells consist primarily of blue-green algae and bacteria, while the remaining organisms are classified as eukaryotic, including other forms of algae.

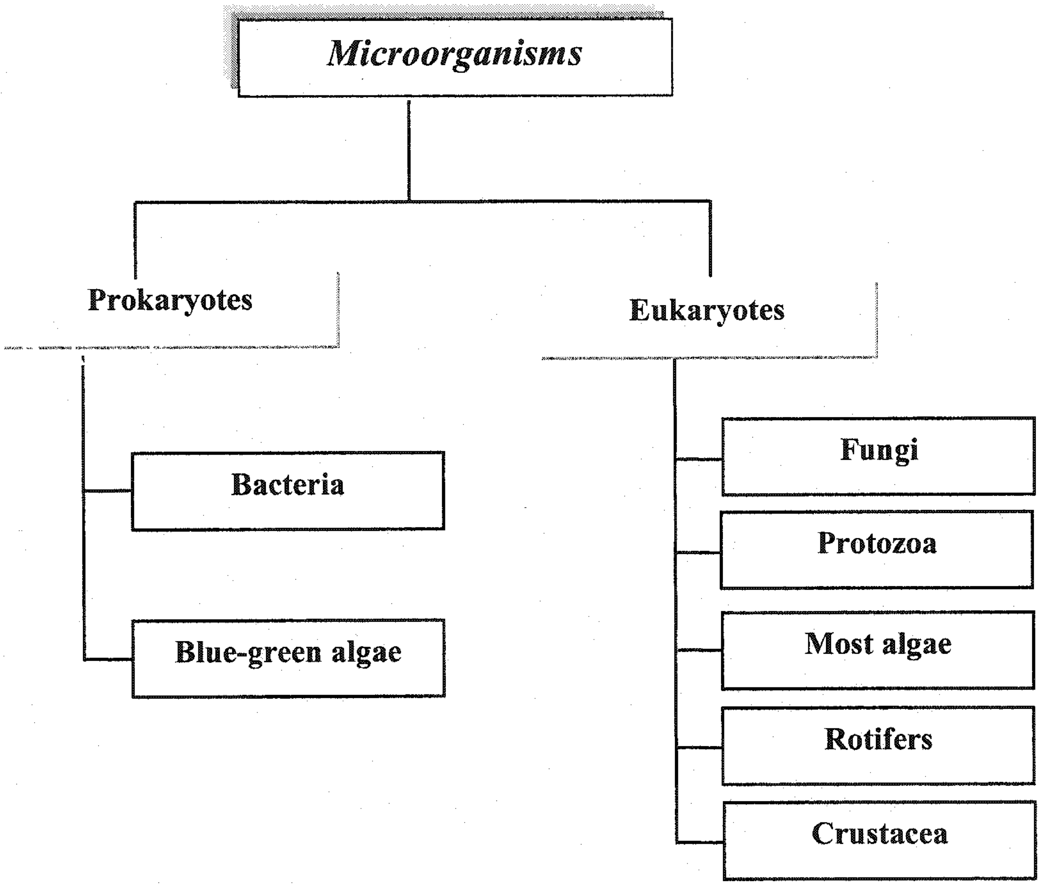


Figure 4.1: Microbial divisions according to cell type (Cookson, 1995)

4.1.1 Bacteria

The bacteria are a large and highly diverse group of prokaryotic organisms. As a result, they are the primary decomposers of organic material in wastewater. They are single-celled organisms and they must have their food in soluble form. Bacteria may occur as single cells, as clusters of cell or in chains made up of cells. Although they may link into clusters or chains, each cell is an independent organism that carries all the necessary life functions. Bacteria can be classified according to the energy and material sources that they require. Table 4.1 describes briefly the classification of bacteria. Particularly, heterotrophic bacteria are the most important species in the degradation of organic material, and they are often classified into three groups. Table 4.2 summarizes the groups of heterotrophic bacteria.

Table 4.1: Classification of bacteria (Henze *et al.,* 1997)

Types of bacteria	Description
Autotrophs	Derive both energy and material from inorganic sources
Heterotrophs	Obtain both energy and material from organic compounds
Phototrophs	Utilize sunlight for an energy source and inorganic substances for a material source

Table 4.2: Classification of heterotrophic bacteria (Henze *et al.,* 1997)

Heterotrophic bacteria	Description
Aerobic heterotrophs	Require oxygen in their metabolic processes
Anaerobic heterotrophs	Make use of organics in the absence of oxygen
Facultative heterotrophs	Act as aerobes when oxygen is present but change to anaerobic processes when oxygen is not available

4.1.2 Fungi

Fungi are heterotrophic and have developed a highly distinctive biology. As indicated by Ramalho (1983), they are useful in biological treatment since they are effective in a wide pH range (typically 2 to 10). According to present knowledge, they comprise numerous species distributed in the following three classes:

1. Phycomycetes
2. Ascomycetes
3. Basidiomycetes

The Phycomycetes are characterized by the absence of septa in the mycelium and they can be subdivided into different groups with respect to their reproduction mechanism. Ascomycetes and Basidiomycetes are higher forms of fungi. Ascomycetes are usually found on decaying bread or fruit. Basidiomycetes mainly appear on decaying trees and mushroom. Ascomycetes and Basidiomycetes are ratified to have little importance in water, but they are highly active in soil. Also, Basidiomycetes can be used in the treatment of hazardous wastes in soil.

4.1.3 Protozoa

Henze *et al.* (1997) pointed out that protozoa are single-celled organisms which reproduce by binary fission. Unlike bacteria, protozoa ingest solid organics as their food sources. They also diet on bacterial cells and colloidal organics. There are a number of aquatic species of protozoa, which are similar to heterotrophic bacteria, deriving both energy and material for growth and reproduction from the same organic food source.

4.1.4 Algae

Algae are autotrophic and photosynthetic organisms (Cookson, 1995). There are thousands of species of algae of various sizes, shapes, and colors in watercourse. Algal cells may be found in clusters, in long filaments attached to banks or bottom material, or remain as single cells suspended in water. Algae have been proven to be ecologically harmful to other inhabitants. In the presence of sunlight, algae metabolize the waste products of heterotrophic bacteria such as CO_2, NO_3^-, and PO_4^{3-} while getting energy from sunlight and increase the pH values of the water. When sunlight is unavailable, algae use stored food for energy and takes off oxygen in the process.

4.1.5 Rotifers and crustacea

Rotifers and crustacea are lower-order organisms that prey on bacteria, protozoa and algae. They help maintain a balance in the populations of primary producers and serve as an important link in the chain by which organic materials are passed on to higher-order organisms.

4.2 Microbial growth kinetic

Microbial growth is essential to the successful operation of all biological wastewater treatment systems since biomass are the primary agents to remove unwanted matter in wastewater. The relationship among organism growth rate, substrate concentration and utilization rate, and cell yield can be used for the design of a full-scale biological wastewater plant. Several environmental factors such as temperature, pH, dissolved oxygen, nutrients and trace elements, and toxicity by organic and inorganic chemicals can have significant influence on the growth of microorganisms.

The rate of increase in microbial concentration in a culture has commonly been expressed in terms of the rate of substrate removal. Typically, the rate of substrate removal that occurs in biological processes can be measured by two basic approaches. The first approaches employs the Michaelis-Menten equations and the second approach applies the Monod relationship for microbial growth.

4.2.1 Michaelis-Menten approach

The Michaelis-Menten equation is most widely used to describe the utilization of substrate by microorganisms. Noticing that most enzyme-catalyzed reactions involving a single substrate are zero order in high substrate concentration, but are first order in relatively low substrate concentration, the Michaelis-Menten equation gives the relationship between the rate of fermentation product production (dP/dt) and substrate concentration, S (Henze *et al.,* 1997). The rate of product production may also be considered as the specific rate of product production, (1/X(dP/dt)), where X is the cell mass concentration. Since the specific rate of product production is proportional to specific rate of substrate utilization, it may be incorporated into the Michaelis-Masten equation to form (Reible, 1998):

$$\boxed{\frac{1}{X}\frac{dS}{dt} = \frac{k_s S}{K_m+S}}$$

Where

$(1/X)(dS/dt)$ = specific rate of substrate utilization [mass/(mass microbes x time)]

dS/dt = rate of substrate utilization [mass/(volume x time)]

k_s = maximum rate of substrate utilization [mass/(mass microbes x time)]

K_m = substrate concentration when the rate of utilization is half the maximum rate [mass/volume]

S = substrate concentration [mass/volume]

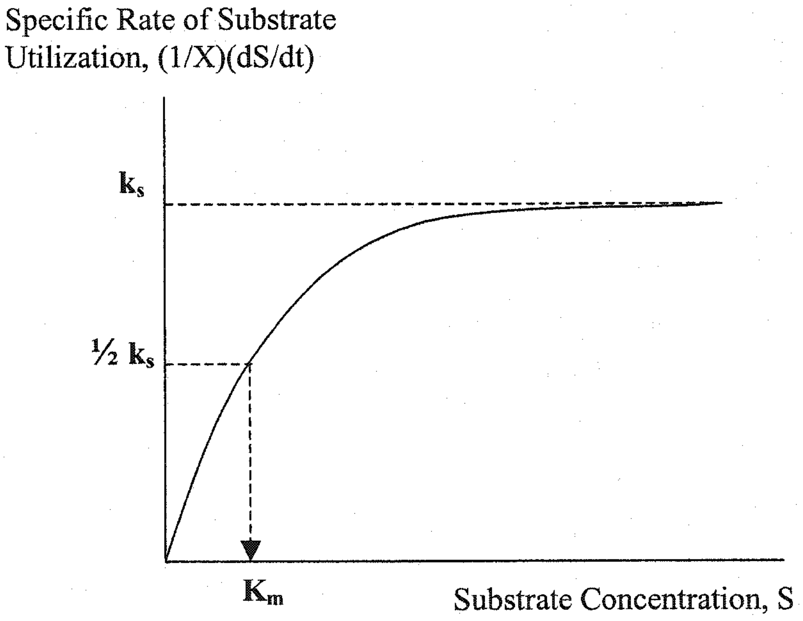


Figure 4.2: Relationship between the specific rate of substrate utilization and the substrate concentration (Reible, 1998)

Figure 4.2 shows the rate of substrate utilization versus the substrate concentration for the Michaelis-Menten relationship. In examining the Michaelis-Menten equation, it indicates that there are two limiting cases. First, if S is relatively large, K_m is negligible in the denominator because it becomes very small in comparison with S. According to Henze *et al.* (1997), the reaction is, therefore, zero-order in the substrate and yields the equation as follows:

$$\boxed{\frac{1}{X}\frac{dS}{dt} = k_s = K}$$

where $k_s = K$ = rate constant for a zero order reaction.

If, on the other hand, S is very small, it can be neglected in the denominator. As a result, the Michaelis-Menten equation turns into as follows (Henze *et al.*, 1997):

$$\boxed{\frac{1}{X}\frac{dS}{dt} = \frac{k_s\,(S)}{K_m} = K\,(S)}$$

where K is the rate constant for a first order reaction in substrate.

To summarize the approach using Michaelis-Menten concept, high substrate concentration is zero order in substrate utilization, and low substrate concentration gives first order in substrate utilization (Henze *et al.*, 1997). To quote an example, when it occurs in laboratory batch culture experiment, the substrate utilization is zero order for a period after the inoculation and after a while becomes first order reaction.

4.2.2 Monod approach

The Monod equation has been used in engineering since 1950s, and it relates microbial growth to substrate concentration (Ramalho, 1983). Monod observed that the microbial growth could be expressed as:

$$\boxed{\frac{dX}{dt} = \mu X}$$

where

dX/dt = rate of cell production [number or mass/time]

X = number or mass of microbes present in substrate

μ = growth rate constant, time $^{-1}$

The growth rate constant, μ, can be obtained by the Monod equation as follows (Warren Viessman and Mark, 1998):

$$\boxed{\mu = \frac{S\,\mu_{max}}{K_s + S}}$$

where

μ_{max} = maximum value of the growth rate constant, time $^{-1}$

S = substrate concentration in solution [mass/volume]

K_s = substrate concentration when the growth rate constant is half the maximum rate constant, [mass/volume]

Figure 4.3 illustrates the relationship between the growth rate constant, μ, and the substrate concentration, S.

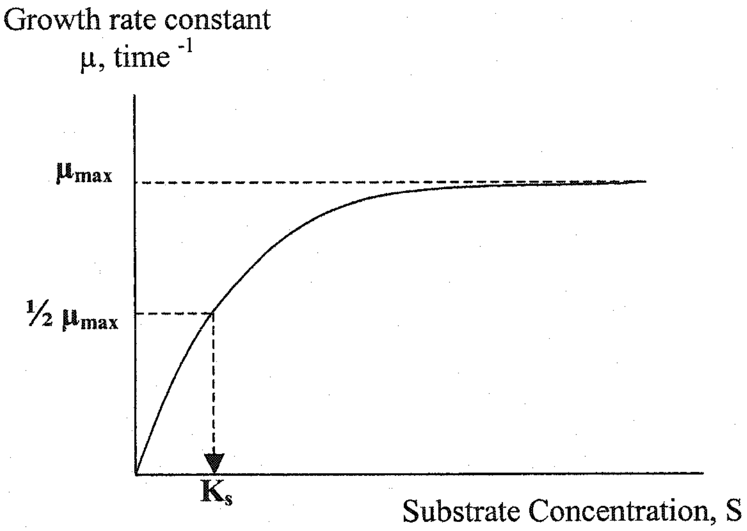


Figure 4.3: Relationship between the growth rate constant and the substrate concentration (Reible, 1998)

The equations displayed above, Michaelis-Menten and Monod, form the foundation for any detailed analysis of biological treatment systems. They have been used to derive design equations for full-scale environmental treatment plants.

4.3 Environmental factors influencing microbial activity

Biological treatment methods take advantages of the ability of microorganisms to degrade organic compounds as a source of energy for their metabolic processes. Those unfavorable constituents are degraded to less objectionable forms and new cells are developed consequently. Many environmental factors upon microbial activity must be taken into consideration, the reasons encompass:

- It is indispensable to maintain biological treatment culture at its optimum activity.
- These environmental factors are significant in evaluating the feasibility of treating certain organic wastewaters by biological treatment processes.
- These factors can affect the effluents from biological treatment processes.

Generally, environmental factors may be widely classified into physical, chemical and biological according to their nature and these factors are illustrated in Table 4.3.

Table 4.3: Environmental factors influencing biological treatment (Ramalho, 1983)

Environmental Factors	Description
Physical	Temperature, total dissolved oxygen, moisture, ultraviolet light
Chemical	pH value, presence of oxidizing and reducing agents, presence of heavy metals, concentration of certain chemicals, electron acceptor
Biological	Energy and substrate sources, toxicity and inhibition, enzymatic processes

4.3.1 Physical environmental factors

Temperature – In biological treatment process, there is one factor that must not be neglected; temperature. Microbial cells and their cell contents are at the same temperature as their environment, and since metabolic reactions are biochemical enzyme-catalyzed reactions, an increase in temperature generally leads to an increase in the rate of reactions until the optimum range is attained. According to Ramalho (1983), microbes can be categorized according to their optimum temperature range as psychrophiles (0 to 10°C), mesophiles (10 to 45°C) and thermophiles (45 to 75°C). Continued exposure to extremely high temperatures (above 75°C) results in cell death. Low temperatures do not cause cell death; however, microbial activity decreases and eventually ceases if the temperature falls below 0°C.

A simplified equation based on the Arrhenius temperature relationship is shown below (Peavy, 1985):

$$\boxed{\frac{k_{T2}}{k_{T1}} = \theta^{T2-T1}}$$

where

k_{T1} = reaction rate constant at temperature T1, °C

k_{T2} = reaction rate constant at temperature T2, °C

θ = temperature correction coefficient

T1, T2 = temperature, °C

Typically, raising the temperature 10°C doubles the reaction rate, and θ ranges from 1.01 to 1.09 (Peavy, 1985).

Total Dissolved Oxygen – Dissolved oxygen must be present in the biological treatment of wastewater for aerobes and for facultative anaerobes while the aerobic process is applied. Suppose that the total dissolved oxygen varies widely, the microbes in the treatment process will have difficulty adapting the environment and subsequently decline, or cease activity. Usually, a concentration of 2 mg/L of total dissolved oxygen is used as a design value for an aerobic reactor (Reynolds & Richards, 1996).

Moisture – Moisture is required for biodegradation for cellular growth since cellular tissue is 75 - 80% moisture (Mulligan 2002). Secondly, moisture is required because it provides a medium for the movement of the microorganisms to the substrate (or vice-versa) for non-motile species. In the case of wastewater treatment, moisture is not an issue. In soil systems, biodegradation can occur at levels well below saturation. Reible (1998) indicated that bacteria fail to grow if the water content of the medium falls below 92% relative humidity. It is generally accepted that the minimum moisture content in contaminated soil is 40% of saturation.

Ultraviolet light – The UV light is toxic to microorganisms at low intensity. In lakes or reservoirs, the UV light from the sun help to kill bacteria. Nevertheless, it is negligible in the activated sludge process. High intensity UV light has been utilized to disinfect drinking water in ships and to treat effluents from biological reactors.

4.3.2 Chemical environmental factors

pH – Microbial activity and substrate utilization as well as biomass growth are dependent on the pH value of the system. Henze *et al.* (1997) mentioned the optimum pH range for bacteria growth is 6.5 to 9. Die-off occurs below a pH of 4 and above 9.5. Microbial activity can alter the pH in the system. For instances, anaerobic treatment converts organic waste to organic acids lowers the pH, and since carbon dioxide is one of the end products produced in aerobic biodegradation, pH is decreased in the reactor. In order to maintain optimum pH range, buffering system is necessary for assisting in neutralization.

Oxidizing & Reducing reagents – The existence of strong oxidizing or reducing reagents are detrimental to microbes at relatively low concentration. Particularly, if the halogen (chlorine, fluorine, bromine, and iodine) has a valence above its lowest valance state, it becomes very toxic to microorganisms since it is a very strong oxidizing agent.

Heavy metals – Heavy metal ions and salts are harmful to microorganisms even at a relatively low concentration. The heavy metals may be found in wastewater include mercury, arsenic, lead, zinc, cadmium, copper, barium, and nickel.

Industrial chemicals – Some industrial chemicals such as organic acids, alcohols, ethers, phenol, chlorophenol, and dyes are persistent to microbial activity if present in adequate concentration. Antibiotics that are produced by pharmaceutical fermentation are poisonous to microbes in small concentrations. Greases, which are not uncommon to be encountered in wastewater, coat the microbes in activated sludge and interfere with their aerobic respiration.

Electron Acceptor - Catabolism typically involves a transfer of electrons from the waste to an electron acceptor. In aerobic systems, bacteria utilize oxygen an acceptor of electrons removed from oxidized organism chemicals. During biotransformation, oxygen is added to the organic molecule and hydrogen is removed, thereby, converting an alcohol to an organic acid. Complete mineralization involves the reduction of oxygen to water and organic carbon is oxidized to carbon dioxide. Due to the low solubility of oxygen in water (solubility of oxygen in water is in the range of 8 mg/L), it must be supplied during aerobic treatment (Henze *et al.,* 1997).

Anaerobic processes do not use oxygen as the terminal electron acceptor. Systems using anaerobic processes consist of a complicated system of reactions resulting in the conversion of hydrolyzed organics to higher organic acids. The acids are degraded to acetic acid, hydrogen and carbon dioxide, which is then metabolized by methanogenic bacteria to produce methane. The terminal electron acceptor during anaerobic respiration typically includes several inorganic, oxygen bearing compounds:

1. **Nitrates.** Reduction to nitrogen; sometimes referred to as anoxic
2. **Sulphates.** Reduction to hydrogen sulphide
3. **Carbon Dioxide.** Reduced to methane via methanogenesis

4.3.3 Biological environmental factors

Energy/Substrate source - Microorganisms require a suitable energy and substrate source in order to sustain their metabolism. The classification of microorganisms is directly based upon the sources from which they derive their energy and cellular carbon, and it is shown above. Fungi and other higher forms of organisms are effective in specific applications. Nevertheless, the inherent use of organic carbon and the oxidation-reduction reactions that occur, make heterotrophic bacteria highly useful in the degradation of organic chemicals originating from residential, agricultural and industrial sectors.

Toxicity and inhibitory effects are observed when the concentration of the organic waste material increases past a certain threshold. An organic substance that is biodegradable at one concentration may be persistent (by inhibiting the growth of biomass) or toxic (eliminating the microbial population) at higher concentrations. This is due to the degree at which the substrate overwhelms the enzymatic systems that degrade it. Similarly, if the concentration is too low, the carbon or energy source may not be sufficient to sustain a microbial population. When designing a biological treatment system, the concentration of organic waste material and its possible toxic or inhibitory effects must be considered.

Enzymatic Processes - Effective degradation of organic substances by microorganisms is dependent on the release of enzymes and the proper contact between substrates and the organisms. Enzymes are large protein molecules composed primarily of amino acids twisted into complex shapes by peptide links and hydrogen bonding. The characteristics of enzymes in biodegradation is two-fold:

1. **Lowering of activation energy.** Enzymes lower the activation energy required for a given reaction to occur. This increases the rate of reactions and facilitates faster biodegradation.

2. **Evolution-induced stereochemistry.** Enzymes have a structural affinity for substrates and therefore can mediate the degradation of particular organic compounds.

3. **Not consumed in reactions.** Enzymes function as catalysts are not directly used up in reactions and can therefore be reutilized for ongoing degradation.

A myriad of different enzymes, not necessarily from the same microorganism, typically acts in a sequential manner to degrade organic waste into simpler compounds (i.e. degradation of TCE or chlorobenzoate). Each enzyme has a particular specificity for different organic compound, which tends to vary widely. Successful degradation, therefore, requires the correct substrate coupled with the release of the appropriate enzyme.

CHAPTER 5

MATERIALS AND METHODS

5.1 Introduction

This experiment was mainly composed of two parts. The first was a batch experiment, and the second one was a continuous flow system. Since not all biomass can undergo the Anammox process, one of the main goals of this study was to demonstrate the feasibility that the given biomass could grow under this condition. It was known that the start up of this process would be rather difficult, so a series of batch tests were performed. From the results of the batch experiments, the major parameters for the continuous flow system were also determined. This provided a realistic analysis of how an industrial anaerobic reactor would operate and would aid in narrowing down the concentration range at which toxicity inhibits degradation.

5.2 Acclimation of the biomass

At the beginning, glucose solution was chosen to be the substrate to replace real wastewater. One gram of glucose, 10 mL of defined media, 5 ppm of NH_4^+ as well as NO_2^- solution were added to the 2L biomass once every two days. Additionally, 1 g of yeast was supplied once a week to acclimate the biomass. The composition of defined media is presented in Table 5.1 and this formulation was provided by SNC – Lavalin Group. Since it was found that the defined media was too concentrated, it was diluted 10 times to ensure that it did not have a negative effect on the sludge. The value of pH in the defined media was then adjusted to 7 by using hydrochloric acid (HCl).

Table 5.1: Composition of defined medium

Element Supplied	Chemical	Quantity (mg/kg COD)	Quantity (mg) required for different COD concentration (mg/L) (sample calculation)	
			100	**500**
N	$(NH_4)_2CO_3$	5650	0.565	2.825
P	$(NH_4)_2HPO_4$	1500	0.15	0.75
Vitamins	Yeast Extract	10000	1	5
Al	$Al_2(SO_4)_3\ 2H_2O$	1.9	0.00019	0.00095
Ca	$CaCl_2\ 6H_2O$	693.8	0.06938	0.3469
Co	$CoCl_2$	4.02	0.000402	0.00201
Cu	$CuCl_2$	0.6	0.00006	0.0003
fe	$FeCl_3\ 6H_2O$	482.1	0.04821	0.24105
Mg	$MgSO_4\ 7H_2O$	2563.5	0.25635	1.28175
Mn	$MnSO_4\ H_2O$	3.1	0.00031	0.00155
Mo	$(NH_4)_6Mo_7O_{24}\ H_2O$	0.2	0.00002	0.0001
Ni	$NiCl_2\ 6H_2O$	2	0.0002	0.001
Zn	$ZnCl_2$	6.3	0.00063	0.00315

5.2.1 Observations

After a few weeks of acclimation, it was observed that the sludge had deteriorated and some of the biomass was broken down into small pieces. Furthermore, the biomass that accumulated in the reactor was noticed to have some milky foam formed as wall growth. The sludge was getting less granular and it was noticed that a layer of dark precipitate had appeared on the top of the biomass. This precipitate was suspected to be iron sulfide (FeS). Owing to the wall growth, the distribution of the sludge over the reactor was not homogeneous. Moreover, the sludge deterioration resulted in almost complete loss of biomass activity. The deterioration of the sludge granules could be related to a microbial population shift. In fact, the biomass was obtained originally from a cheese factory. The mineral medium used in this experiment consisted mainly of glucose, ammonium, and nitrite. After inoculation, the anaerobic ammonium oxidizing population grew, but a large amount of other biomass starved. According to Van Loosdrecht and Jetten (1998), when the growth and starvation process of the different populations were not attuned, sludge deterioration could result.

Using glucose for acclimation might not be a proper carbon substrate for the microorganisms. For this reason, evaporated milk was supplied to the reactor as a replacement of the glucose solution. Once again, 10 mL of defined media, 5 ppm of NH_4^+, as well as NO_2^- solution were put into a bottle of 2 L biomass once every two days. Instead of utilizing glucose, 10 mL of evaporated milk was added every 2 days. After three weeks of acclimation, it was seen that wall growth was no longer present and the sludge stayed in its granular shape. Also, the dark precipitate previously mentioned no longer existed. This showed that evaporated milk was a better option to acclimate this particular sludge from the cheese factory.

After 60 days, the color of the sludge granules had changed from brownish to reddish. This phenomenon was described in a study by Mulder *et al.* (1995) as well. According to Mulder *et al.* (1995), the ammonium oxidizing organisms (irregular, coccoid, gram – negative bacterium) had become the dominant organism in a denitrifying fluidized bed reactor. They also indicated that the color change and expansion of the biomass represented the growth of new sludge granules.

5.3 Experimental set up

It is important to note that ammonium ion oxidization can also occur due to other conversions during Anammox process. As a result, the ammonium ion measurement can be distorted. In order to achieve better results, control samples were used for the following experiments.

Control samples, in this research, consisted of the defined media, substrates as well as the biomass but without nitrite (NO_2^-). Since there is no nitrite addition, its volume was filled with distilled water. Therefore, the control served to correct the measurements. Also, the difference between the control and the specimens with nitrite (NO_2^-) addition would be the true amount of ammonia reduction due to the Anammox process.

5.3.1 Batch experiment

There were three sets of serum bottles. One set of serum bottles was intended for COD : N = 150 : 1, another set with COD : N = 75 : 1 and a third set was for the control samples. Figure 5.1 shows the schematic representation of the setup. Fifty mL of defined medium was mixed with the glucose solution in each set at the beginning so that the theoretical COD levels could be controlled by adding glucose. Afterward, the bottles were inoculated with 50 mL of biomass and 50 mL of the defined media was supplied to the serum bottles. Subsequently, different concentrations of nitrite are poured and butyl-rubber stoppers are inserted into the serum bottles with aluminum seals to prevent the entry of air. In order to attain anaerobic conditions, the samples were flushed with nitrogen gas for 2 minutes after sealing. The operating temperature was room temperature and the initial pH of the defined media was kept at 7.

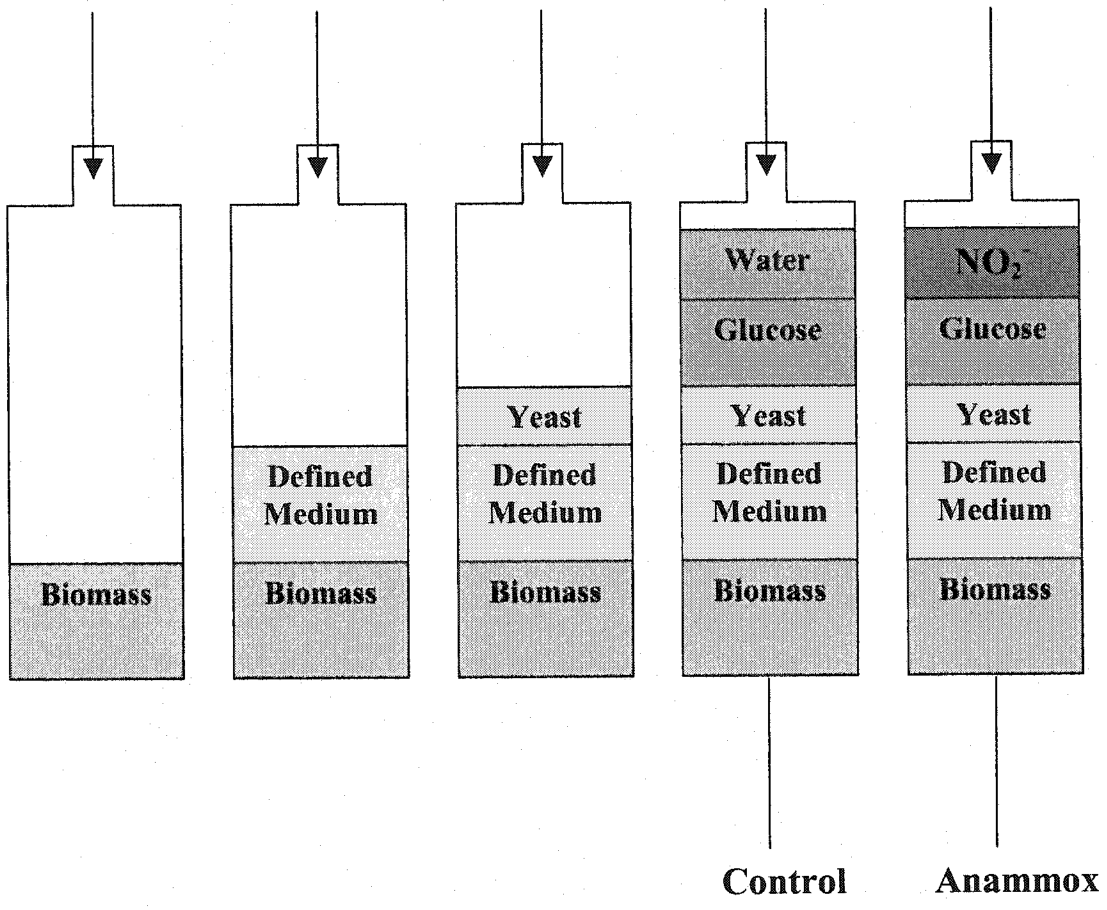


Figure 5.1: Schematic representation of batch experiments

All assays were carried out in a 100 mL total working volume. Each batch experiment was done in duplicate. COD concentration, ammonia as well as nitrite level were then measured at *t* = 24 hours and *t* = 48 hours (the procedures are described in the upcoming section 5.4). Media compositions are summarized in Table 5.2 and supplementary details are provided subsequently.

Table 5.2: Summary of media content in the serum bottles

	Substrate	Sodium Nitrite ($NaNO_2$)	Expected Nitrite concentration	Expected COD : N ratio	Variation of parameter
Case no. 1 (a)	1 g glucose & 0.1 g yeast	0 g	Control	75 : 1	Room Temperature & pH = 7
Case no. 1 (b)	1 g glucose & 0.1 g yeast	0.5 g	$NO_2^- - N = NH_4^+ - N$	75 : 1	Room Temperature & pH = 7
Case no. 1 (c)	1.5 g glucose & 0.1 g yeast	1 g	$NO_2^- - N = 2\,NH_4^+ - N$	75 : 1	Room Temperature & pH = 7
Case no. 2 (a)	2 g glucose & 0.1 g yeast	0 g	Control	150 : 1	Room Temperature & pH = 7
Case no. 2 (b)	2 g glucose & 0.1 g yeast	0.5 g	$NO_2^- - N = NH_4^+ - N$	150 : 1	Room Temperature & pH = 7
Case no. 2 (c)	2.5 g glucose & 0.1 g yeast	1 g	$NO_2^- - N = 2\,NH_4^+ - N$	150 : 1	Room Temperature & pH = 7
Case no. 3 (a)	1 g glucose & 0.1 g yeast	0 g	Control	75 : 1	Temperature = 35°C & pH = 7
Case no. 3 (b)	1 g glucose & 0.1 g yeast	0.5 g	$NO_2^- - N = NH_4^+ - N$	75 : 1	Temperature = 35°C & pH = 7
Case no. 3 (c)	1.5 g glucose & 0.1 g yeast	1 g	$NO_2^- - N = 2\,NH_4^+ - N$	75 : 1	Temperature = 35°C & pH = 7
Case no. 4 (a)	2 g glucose & 0.1 g yeast	0 g	Control	150 : 1	Temperature = 35°C & pH = 7
Case no. 4 (b)	2 g glucose & 0.1 g yeast	0.5 g	$NO_2^- - N = NH_4^+ - N$	150 : 1	Temperature = 35°C & pH = 7
Case no. 4 (c)	2.5 g glucose & 0.1 g yeast	1 g	$NO_2^- - N = 2\,NH_4^+ - N$	150 : 1	Temperature = 35°C & pH = 7
Case no. 5 (a)	2 g glucose & 0.1 g yeast	0 g	Control	150 : 1	Temperature = 35°C & pH = 6
Case no. 5 (b)	2 g glucose & 0.1 g yeast	0.5 g	$NO_2^- - N = NH_4^+ - N$	150 : 1	Temperature = 35°C & pH = 6

First set: In the first assay, 1 g/ 100 mL of glucose as well as 0.1 g/100 mL of yeast were used in an effort to obtain a COD : N ratio of 75 : 1. The ammonium ion concentration of the defined media was measured so as to determine the amount of nitrite being added to the serum bottles. Adding 0.5 g of sodium nitrite ($NaNO_2$) gave the nitrite concentration similar to the ammonium ion concentration of the defined media. Accordingly, doubling the amount of sodium nitrite addition could provide twice the nitrite level as the ammonium ion concentration in the defined media. The first set of samples was tested under the room temperature with pH of 7 and was carried out for 2 days. The control was not supplied with any sodium nitrite.

Second set: Strous *et al.* (1997) have stated that the start up was one of the most crucial aspects for the Anammox process. According to Reible (1998), the COD/N ratio is a significant factor to start up a biological process. Therefore, in the second set of samples, 2 g/ 100 mL of glucose as well as 0.1 g/100 mL of yeast were utilized to make the COD : N ratio equal to 150 : 1. Other media content and physiological parameters were same as the first set for comparison purposes. This set was intended to evaluate whether a higher COD : N ratio would have improved the start up of the Anammox process. This set was conducted in a 2-day period.

Third set: The third set was a repetition of the first set but with a variation in temperature. As pointed out in section 4.3.1, temperature is an important biological factor that must not be ignored. Peavy (1985) indicated that raising the temperature 10°C can double the reaction rate. This set of experiments ran for 2 days at a temperature of 35°C. The main objective for this set was to determine whether the temperature increase would stimulate the organisms in the Anammox process and allow for a good start up under low COD : N ratio concentrations.

Fourth set: Mike *et al.* (1998) proved that the optimal temperature range of the Anammox process was from 30 to 37°C. With a higher COD : N ratio (150 : 1), the fourth set of samples was anticipated to achieve higher ammonium ion removal efficiency. The duration of this experiment was 2 days and the aim of this set was to see whether the conversion rate of ammonium ion and nitrite, as well as COD, might have improved.

Fifth set: The optimum range of pH for the Anammox process was investigated by Mike *et al.* (1998). They concluded in their research that a pH value between 7.0 and 8.5 was good for the Anammox process. In order to evaluate the alkalinity of the system and the effect of pH to the process, the pH value of this fifth set of samples was set to 6. With a COD : N ratio at 150 : 1, this experiment lasted for 2 days and the purpose was to determine if the biomass suffered any inhibitory effects due to the low pH level.

5.3.2 Continuous flow

The BioFlo 110 Modular Benchtop Fermentor was utilized for this experiment as an anaerobic continuously stirred tank reactor. From the study of Van Dongenet *et al.* (2001), the specific growth rate of the microorganisms in the Anammox process is very low (the doubling time of 11 days). Currently available microbiological technologies are not designed to deal with very slowly growing microorganisms. In the present investigation, the sequencing batch reactor (SBR) was proven to be an excellent experimental set up in which the biomass was retained very efficiently (greater than 90%) (Strous *et al.,* 1998). Additionally, Strous *et al.* (1998) showed that a homogeneous distribution of substrates, products and biomass aggregates over the SBR reactor was achieved, and the reactor has been in operation reliably for more than 2 years. Therefore, the SBR was chosen at the beginning of this study in order to enrich the microbial community which oxidizes ammonium ion under anaerobic conditions.

The Anammox population was cultivated and enriched in the SBR for two months. Afterwards, the SBR was filled continuously, and the system changed to a continuously stirred tank reactor. With the anaerobic continuously stirred system, the retention time fell between 24 and 48 hours, depending on the influent and effluent flow rate. The fermentor was a 2.2 L vessel with baffles and equipped with a heat blanket. It was controlled by the New Brunswick Scientific's BioCommand software and the complete representation of the experimental setup is as illustrated in Figure 5.2. The influent was composed of defined media, glucose, yeast as well as nitrite. The initial pH value of the influent was adjusted to 6 with the intention of preventing any dilution error. The working volume was 2 L and the vessel was stirred at 50 ± 5 rpm (using a six-bladed turbine stirrer, with a diameter one – third of the vessel diameter). The temperature was kept at 35°C by the heat blanket and the vessel-cooling coil. Anaerobic condition was obtained by flushing the vessel with nitrogen gas at the beginning of the experiment. The oxidation-reduction potential was monitored using a combined/redox electrode. The gas production in the anaerobic continuously stirred reactor was sufficient to maintain anaerobiosis in the system, so it was not necessary to flush the system with nitrogen gas continuously. A pH probe was installed in order to maintain the pH value in the system at 6.0 ± 0.1. The buffer solution used was 20 mM potassium hydroxide (KOH). Ten ml samples were taken from the effluent every three hours for analytical purposes and the stirrer was stopped for 10 minutes so that the biomass could settle. Ten ml of biomass were also withdrawn from the fermentor bi-weekly to determine the growth of the biomass.

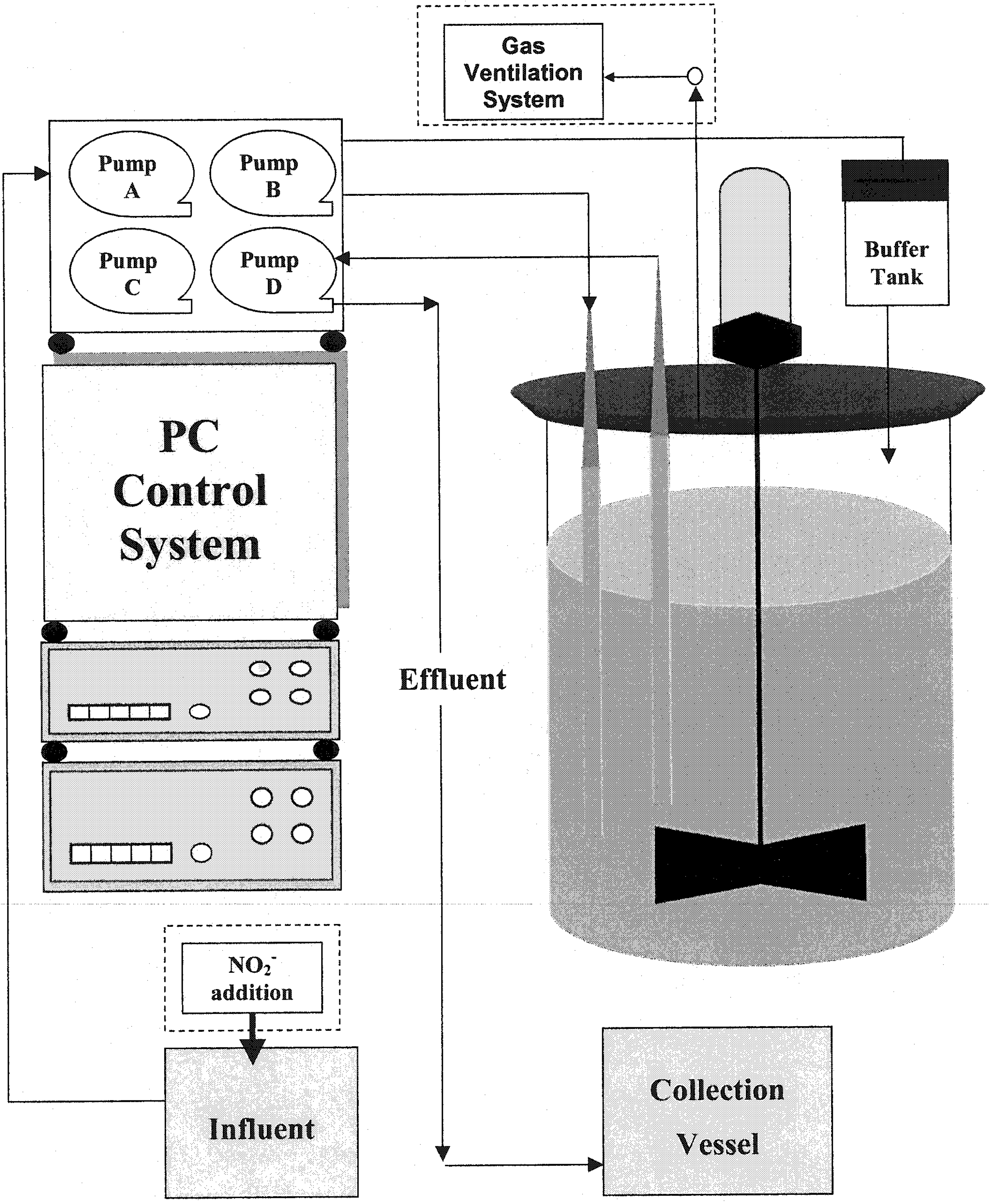


Figure 5.2: Schematic representation of the anaerobic continuously stirred tank reactor

5.4 Analytical procedures

Before beginning the analysis, the samples taken from the batch and continuous flow experiments were filtered by Whatman syringe filter of 0.45 µm pore size, then preserved by refrigeration at 4°C immediately for later analysis. The analytical methods are presented in the following sections.

5.4.1 COD study

COD concentration was measured colorimetrically following the procedures from Rump and Krist (1992). COD reagents were purchased from Fisher's Scientific. One mL of sample solution was added to 4 mL distilled water. Hence, samples were diluted five times before they were mixed with the COD reagent. A 2.5 mL sample was poured into the colorimeter tube in which contains 7.5 mL of COD reagent. Then it was capped and shaken well for a few minutes. The colorimeter tube was then put into a heater for 2 hours at 150 °C. After that, an additional 30 minutes was required for maximum color development. It was necessary to calibrate the solutions of known concentration before analyzing an unknown sample. Therefore, preparation of the standard was performed:

- Low standard = distilled water (0 ppm)
- High standard = 166.7 ppm

Putting the tube into UV visible spectrometer, the actual COD concentration in the sample was determined.

5.4.2 Ammonia level

Corning's Ammonia Combination Electrode was used for the measurement of ammonia concentration. It is designed for the accurate determination of ammonia dissolved in aqueous solutions. This electrode consists of two major components, a pH electrode and a gas permeable membrane mounted on a replaceable module. The ammonia electrode requires calibration in solutions of known concentration before analysis of an unknown sample can be performed. Therefore, preparation of a standard was imperative and three standards were used:

- Low standard = 0.04 ppm
- Sample standard = 0.4 ppm
- High standard = 4 ppm

Samples were made alkaline but only after they had been equilibrated in the acid range. This step converted the NH_4^+ to NH_3. Once the reading was stabilized on the acid side, which usually takes 2 to 5 minutes, 0.5 mL of 10 N sodium hydroxide (NaOH) was injected into 50 mL of test solution and ammonia concentration was obtained.

5.4.3 Nitrite and nitrate concentration

Nitrite and nitrate concentrations were determined by a colorimetric method after a reaction with specific reagents in the kits, which were purchased from Fisher's Scientific. A 5 mL sample was poured into the colorimeter tube. A graduated cylinder was used to measure 5 mL of Mixed Acid Reagent, and then it was added to the tube and mixed with the sample. Afterwards, 0.2 g of the Color Developing Reagent was put into the solution and mixed gently for one minute. Five more minutes were needed for maximum color development. The mixed solution was then analyzed by using UV visible spectrometer. Subsequently, nitrite and nitrate level were obtained.

For the best possible results, a reagent blank was used to account for any contribution to the test result by the reagent system. A reagent blank was created by making use of a distilled water sample. Subtracted the reagent blank from all test results of unknown samples and the true nitrite – nitrogen ($NO_2^- – N$) was found. In order to convert nitrite – nitrogen ($NO_2^- – N$) results to ppm nitrite (NO_2^-), it had to multiply the results by 3.3. Since nitrite interferes at all levels, the equation attached with the kits was used to compensate for nitrite interferences:

$$\boxed{\text{Test result (ppm)} - \text{Nitrite} - \text{N (ppm)} \times 5.5 = \text{true Nitrate} - \text{N reading}}$$

5.4.4 Volatile fatty acids examination

The volatile fatty acid concentration in the system was determined by a Beckman Coulter system gold HPLC (high pressure liquid chromatography). A YMC 8473 column was used for analysis of wastewater samples. The solvent used was 50 mM ammonium phosphate solution with a pH value of 2.4. The flow rate was set at 1 mL/min and the total run time for each test was 20 minutes. The operating temperature was kept at 30°C and the injected volume of each analysis was 10 μL. The wavelength in the detective channel was 210 nm. Preparation of standard mixture was done by adding 1 g of pure acetic acid, propionic acid and butyric acid to 1L of HPLC water to make the standard mixture solution of 1% concentration.

5.4.5 Volatile suspended solids (VSS) analysis

To monitor the growth of the biomass in the continuous flow system, volatile suspended solids were determined. Initially (at $t = 0$), the 2 L SBR was inoculated with 1 L biomass. The defined media was supplied with a hydraulic retention time of 48 hours. Between $t = 0$ and $t = 60$ days, the nitrogen load of the SBR was raised gradually by increasing the influent concentration. In order to obtain volatile suspended solids, total suspended solids were measured.

Total suspended solids - the matter that remained as residue upon evaporation and drying at 105°C for 2 hours and the concentration of total suspended solids was the weight of dry solids divided by the volume of the sample, in mg/L.

Volatile Suspended Solids - the amount of total solids burned off at $550 \pm 50^{\circ}$C for 2 more hours. The concentration of volatile suspended solids was the weight of dry solids on the filter minus the weight of fixed solids on the filter divided by the liquid sample volume.

CHAPTER 6

RESULTS AND DISCUSSION

6.1 Batch test results

6.1.1 Start up of the Anammox process

Six Anammox serum bottles inoculated with the acclimated biomass were started up for this study. In the first instance, synthetic defined media, glucose as well as nitrite were added to the serum bottles. This was to determine whether it was possible to have ammonium ion deduction from the biomass and to find out the amount of nitrite required in the system. Unfortunately, the first few attempts were not successful since no ammonium ion removal was observed (results not shown). The reasons could be due to the existence of oxygen and/or uncorrected COD : N ratio. The literature study indicated that the Anammox activity is only possible under anoxic conditions (Mike *et al.,* 1998). Therefore, the samples were flushed with nitrogen gas for 5 minutes so that all air was removed.

Then, with the adjustment of the COD : N ratio in the system, reduction of ammonium ion was expected. As illustrated in Figure 6.1, at t = 24 hours, a slight ammonia conversion was measured. Figure 6.1 also shows that 26 % of ammonium ion removal was attained with COD : N = 75:1 and 30 % of ammonium ion removal with COD : N = 150 : 1. In both cases, the concentration of ammonium ion decreased during the first 24 hours but increased again at the end of the experiment. In contrast, with double of nitrite addition, Figure 6.2 presents that both cases had very little ammonium ion removal on day 2 and enhancement of ammonium ion concentration on day 3. This illustrated that when a high concentration of nitrite existed in the system, Anammox activity could not start.

Nitrate levels were also measured for both cases; however, low results were obtained (results not shown). This phenomenon may be caused by nitrite interference. Barring the nitrite interference, strong oxidizing and reducing substances would still interfere with the nitrate reading. For those samples that contained high concentrations of iron and copper, low nitrate readings were expected. The final nitrate concentrations on day 3 were approximately 8 mg NO_3^- – N/L.

The increase of ammonium ion after *t* = 24 hours could be due to two reasons. First of all, as indicated in Figure 6.3 and 6.4, more than 80 % of nitrite was removed at the first 24 hours. As a result, ammonium ion oxidation was affected due to the nitrite limitation. Secondly, nitrate was produced during the Anammox process, and the ammonium ion would be increased owing to the conversion of nitrate.

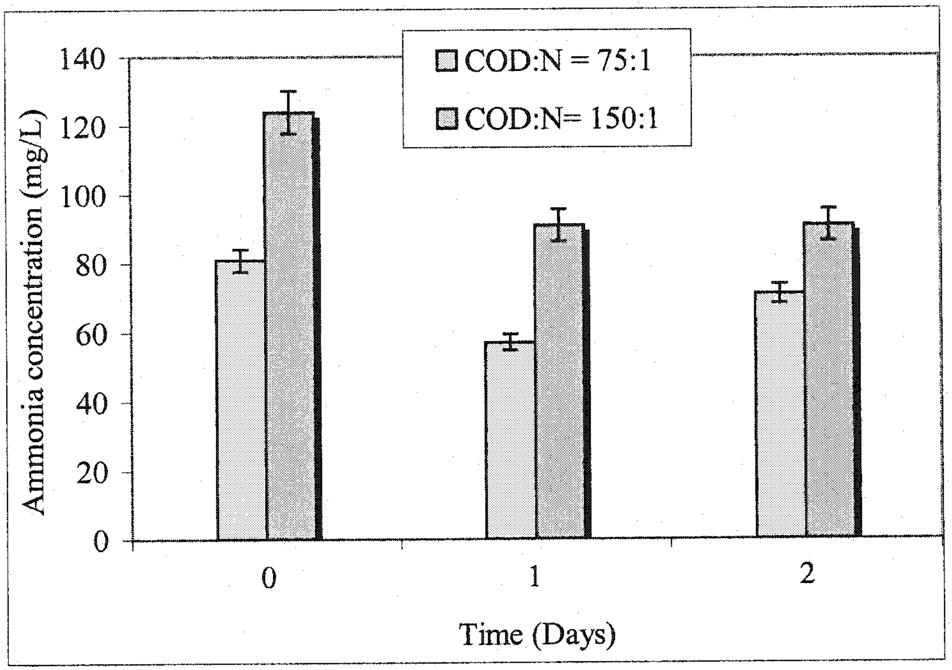


Figure 6.1: Ammonia concentration with NO_2^- addition equal to NH_4^+ concentration

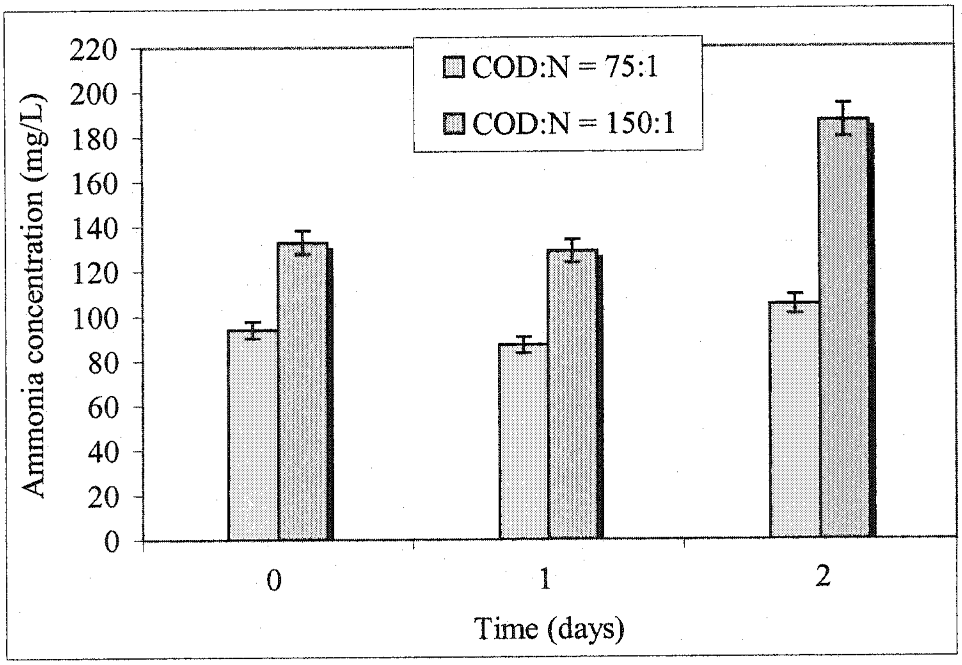


Figure 6.2: Ammonia concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

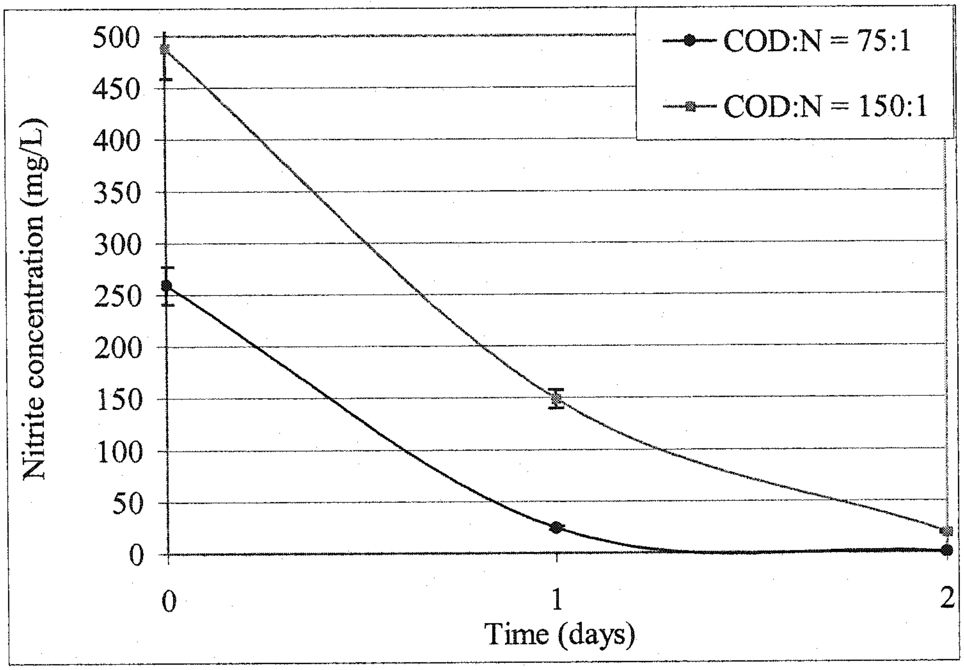


Figure 6.3: Nitrite concentration with NO_2^- addition equal to NH_4^+ concentration

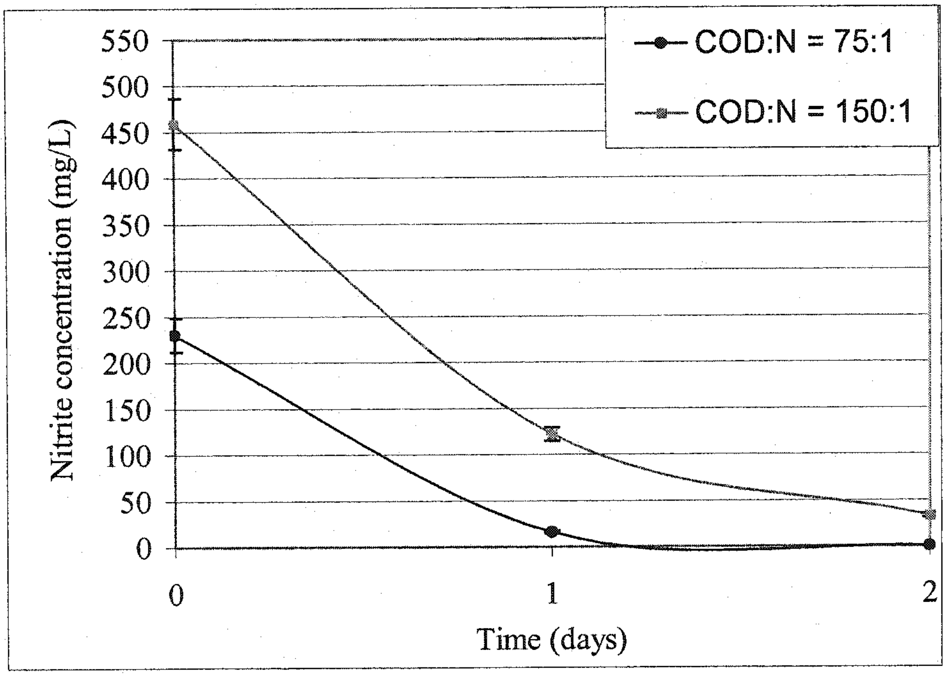


Figure 6.4: Nitrite concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

In order to evaluate the Anammox process, the COD removal was also investigated during this experiment. Figure 6.5 demonstrates that for both cases, COD : N ratio of 75 : 1 as well as 150 :1, approximately 30 % of COD was deducted accompanied with ammonium ion removal in the Anammox process. Nevertheless, in the other cases with higher nitrite addition, Anammox activity failed to start and only 25 % of COD removal efficiency was achieved. Figure 6.6 presents the results. This phenomenon was probably because of the toxic effects due to its high nitrite concentration in the system. From the study of Strous *et al.* (1997), they stated that the Anammox process was inhibited by nitrite at nitrite concentrations higher than 20 mM.

With respect to the ammonium ion removal efficiency, it seems that a COD : N of 150 : 1 gives greater potential to start up the Anammox process. Moreover, the nitrite addition should be similar to the ammonium ion concentration in the system. These points would be verified again in the next experiment.

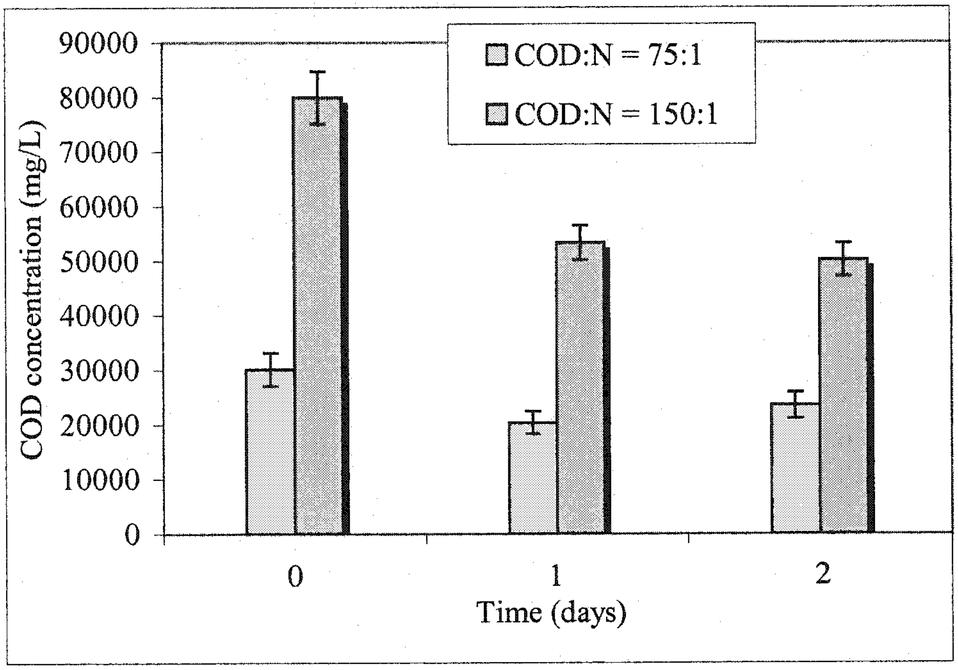


Figure 6.5: COD concentration with NO_2^- addition equal to NH_4^+ concentration

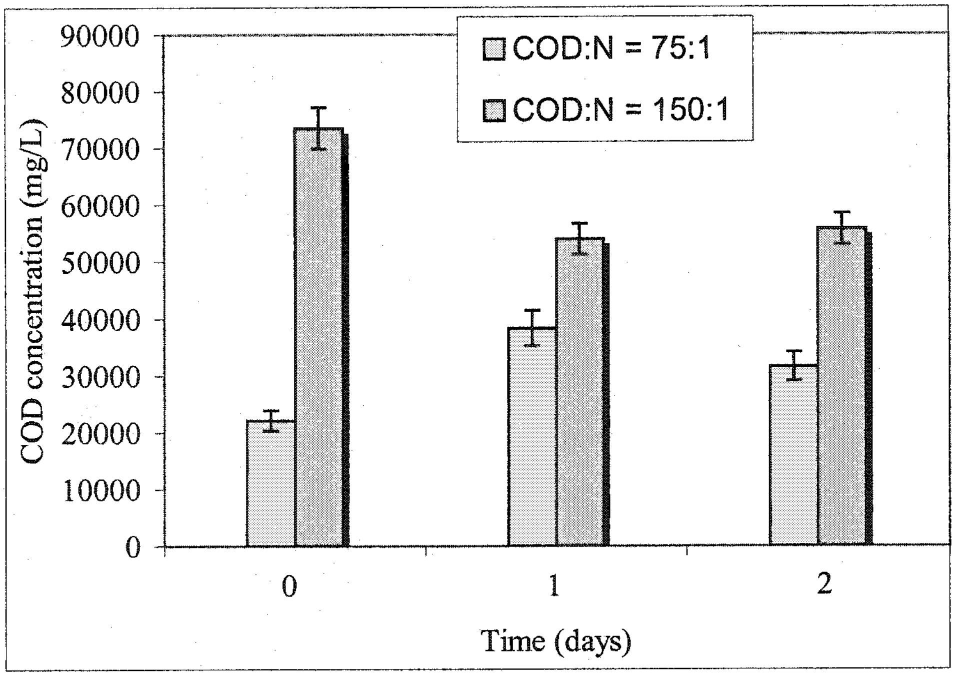


Figure 6.6: COD concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

6.1.2 Biological factor - temperature

As was mentioned previously, the rate of reactions is expected to increase with an increase in temperature (Ramalho, 1983). From the literature review, the optimum temperature for the Anammox process is 30 – 37°C (Van Dongen *et al.,* 2001). Hence, this set of samples was run in an incubator at temperature of 35°C. The addition of defined media, glucose, as well as nitrite was the same as the former set of samples. However, since the acclimated biomass was inactive, the new biomass was inoculated and mixed with the old one.

As shown in Figure 6.7 and Figure 6.9, when the nitrite addition and ammonium ion concentration were close, ammonium oxidation occurred. More than 50 % of ammonium ion and COD removal was attained in the case of COD : N = 150 :1 at $t = 24$ hours. Additionally, Figure 6.11 demonstrates that the nitrite concentration was reduced dramatically during the first 24 hours. This caused the increase of ammonium ion concentration at the end of this experiment. The final nitrate concentrations for both samples on day 3 were in the area of 10 mg NO_3^- – N/L.

Figure 6.7 and Figure 6.9 also shows that in the samples with COD : N = 75 :1, the removal efficiency of ammonium ion and COD was only about 20 %, which was less than the previous test. This phenomenon could be explained by the non-homogenous property of the biomass. As was indicated, the new biomass was added and mixed up with the previous acclimated sludge. During the process, the biomass might not be distributed evenly and the samples with lower COD : N ratio could be inoculated with more new sludge. Since this new sludge was not acclimated under the same conditions as the previous one, its activity was affected due to the high ammonia and nitrite concentrations in the influent. Hence, the Anammox process was influenced during the testing period.

Figure 6.8 illustrates that when nitrite was present at high concentrations for a longer period, the Anammox activity was lost and very little ammonium ion conversion occurred. Even though Figure 6.10 and 6.12 shows that the COD and nitrite concentrations also decreased during the experiment, these results suggest that the Anammox activity was inhibited when higher nitrite addition was used.

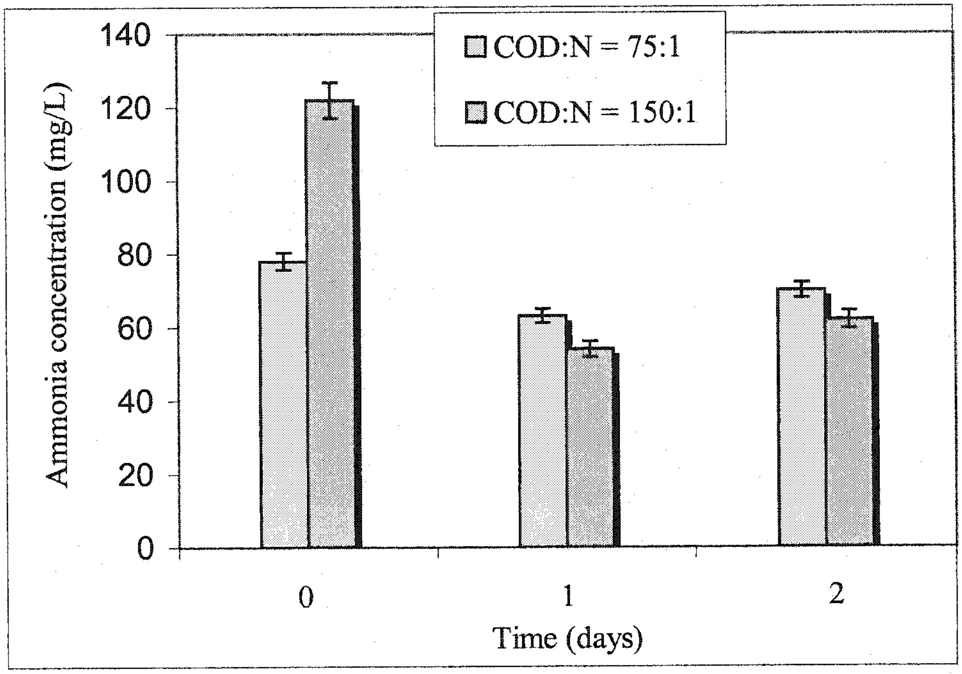


Figure 6.7: Variation of temperature (temperature = 35°C), ammonia concentration with NO_2^- addition equal to NH_4^+ concentration

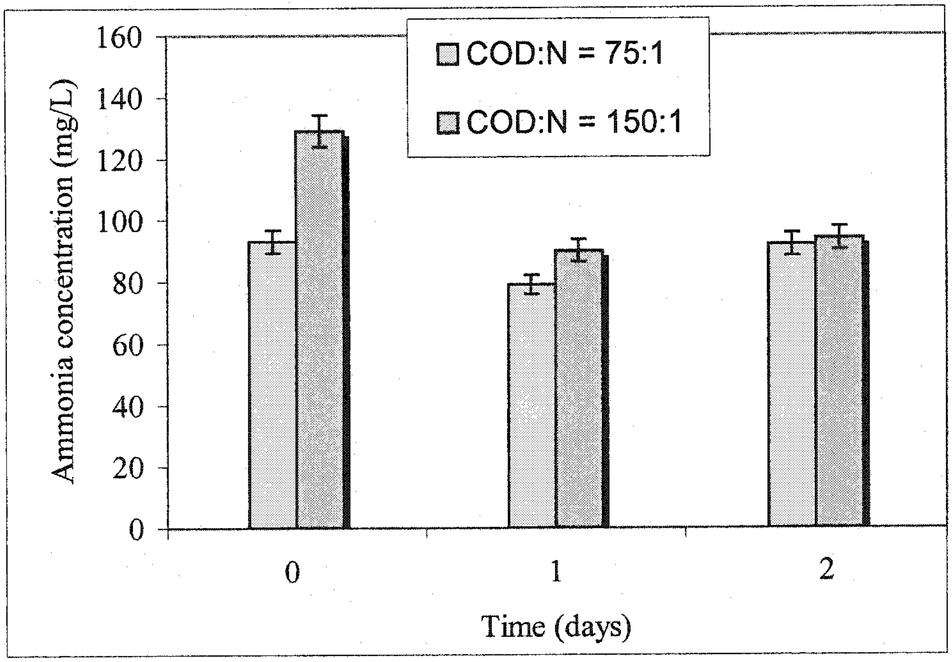


Figure 6.8: Variation of temperature (temperature = 35°C), ammonia concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

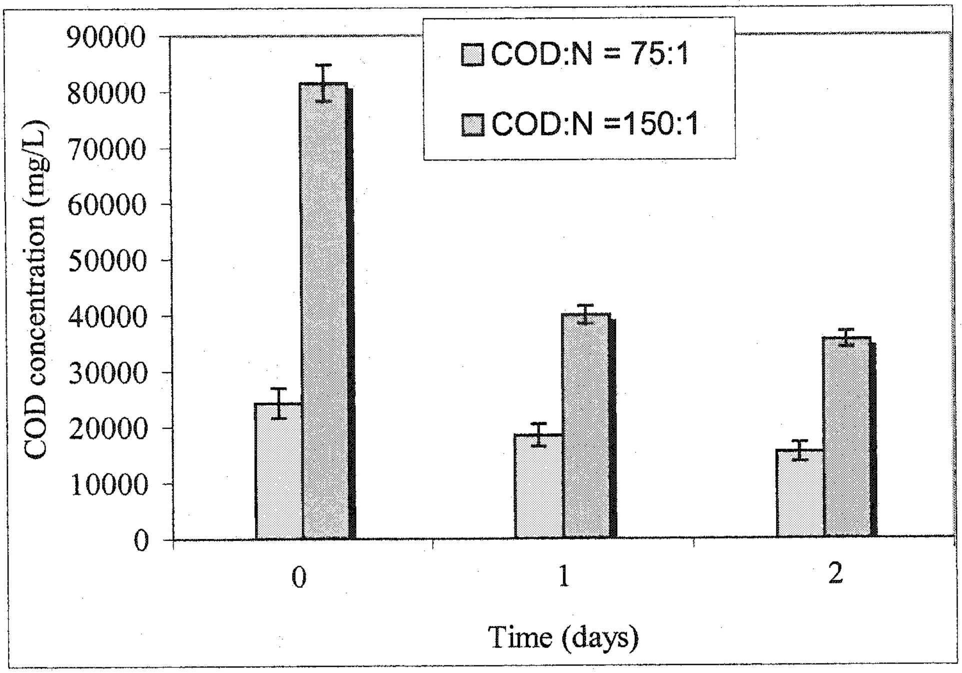


Figure 6.9: Variation of temperature (temperature = 35°C), COD concentration with NO_2^- addition equal to NH_4^+ concentration

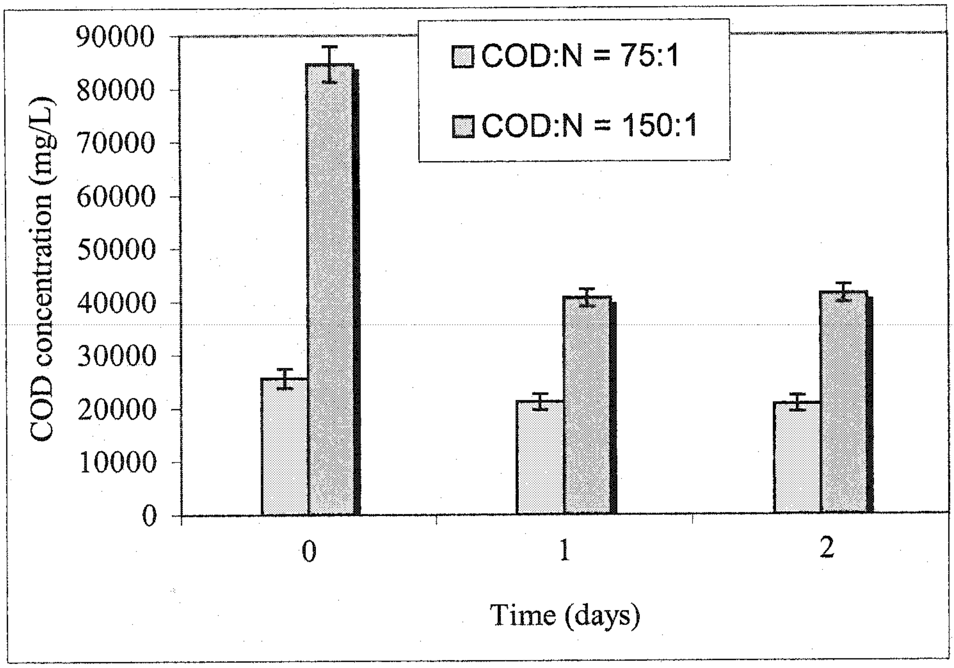


Figure 6.10: Variation of temperature (temperature = 35°C), COD concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

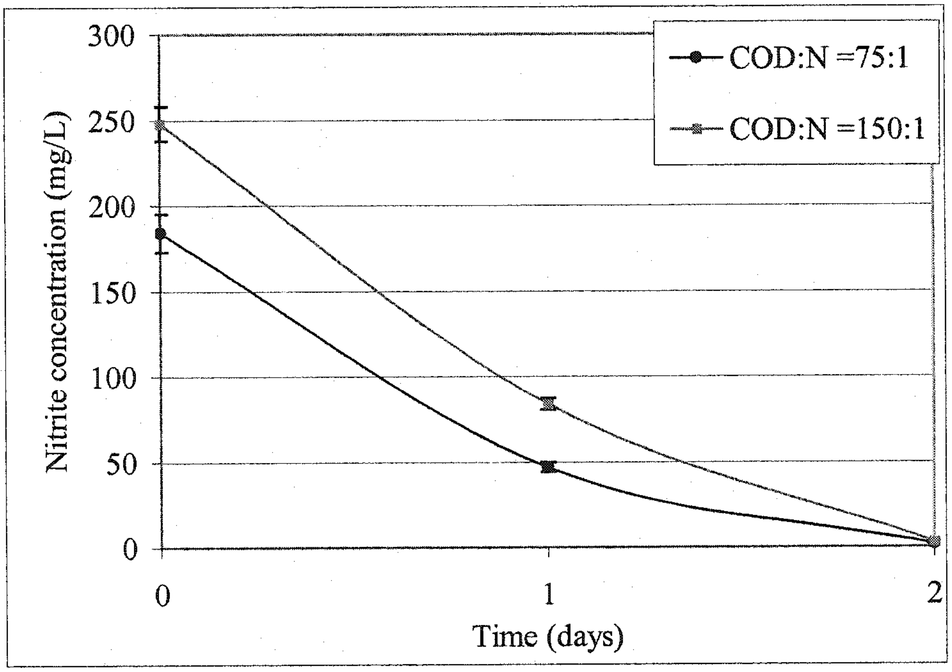


Figure 6.11: Variation of temperature (temperature = 35°C), nitrite concentration with NO_2^- addition equal to NH_4^+ concentration

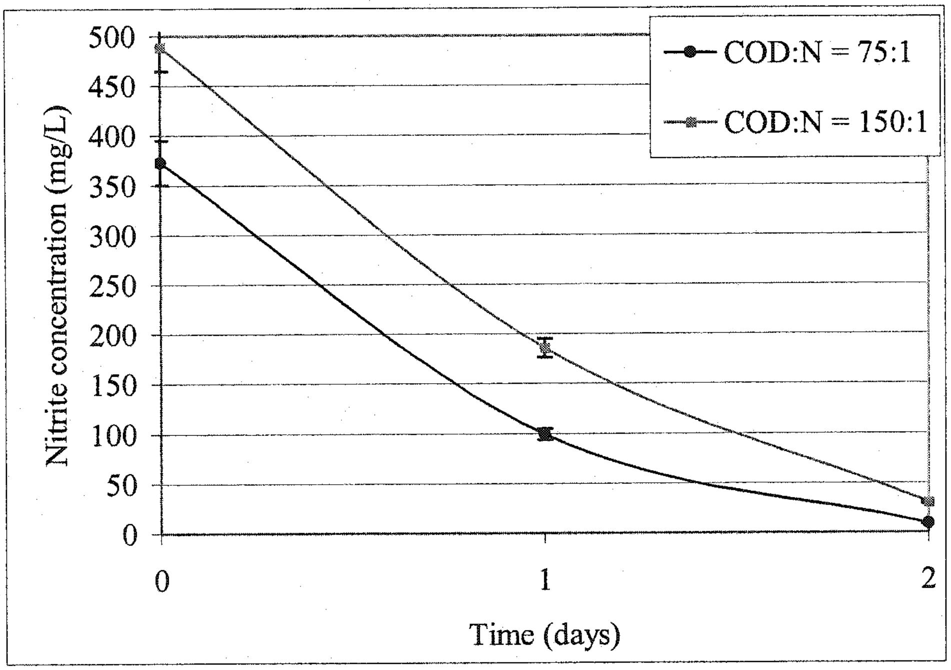


Figure 6.12: Variation of temperature (temperature = 35°C), nitrite concentration with NO_2^- addition equal to 2 times of NH_4^+ concentration

These results proved that nitrite addition should be the same as ammonium ion concentration in the influent so as to have a successful Anammox process. Also, as was expected, the removal efficiency improved with the control of temperature. From these batch studies, a few important points can be concluded:

- COD : N = 150 : 1 provides good start up for the Anammox process.
- Nitrite addition is dependent on the amount of ammonium ion in the system. Ideally, their concentration ought to be equal or similar.
- Temperature is a very important factor in the Anammox and must be monitored during the process.

6.1.3 Biological factor - pH

In the anaerobic process, the pH of the environment within a reactor should range between 6.5 and 8.2 (Mulligan, 2002). Under certain conditions, it may be possible to operate at a pH as low as 6.0 and still make a successful anaerobic process (Speece, 1996). From the study of literature review, the optimum pH range from 7.0 to 8.5. The main goal of this experiment was to evaluate the effect of pH on the Anammox process. After determining the important parameters (as described above in section 6.1.2), they were applied to this set of samples. In this case, on the other hand, the pH of the defined media was adjusted to 6.0 by using hydrochloric acid (HCl).

During the test period, the pH value was monitored and kept at 6.0. Figure 6.13 presents that more than 50 % of ammonium ion removed at t = 24 hours and Figure 6.14 shows 50 % of COD was removed at the end of the experiment. These results revealed that the Anammox sludge was capable of keeping ammonium ion and nitrite removal efficiency at a pH of 6.

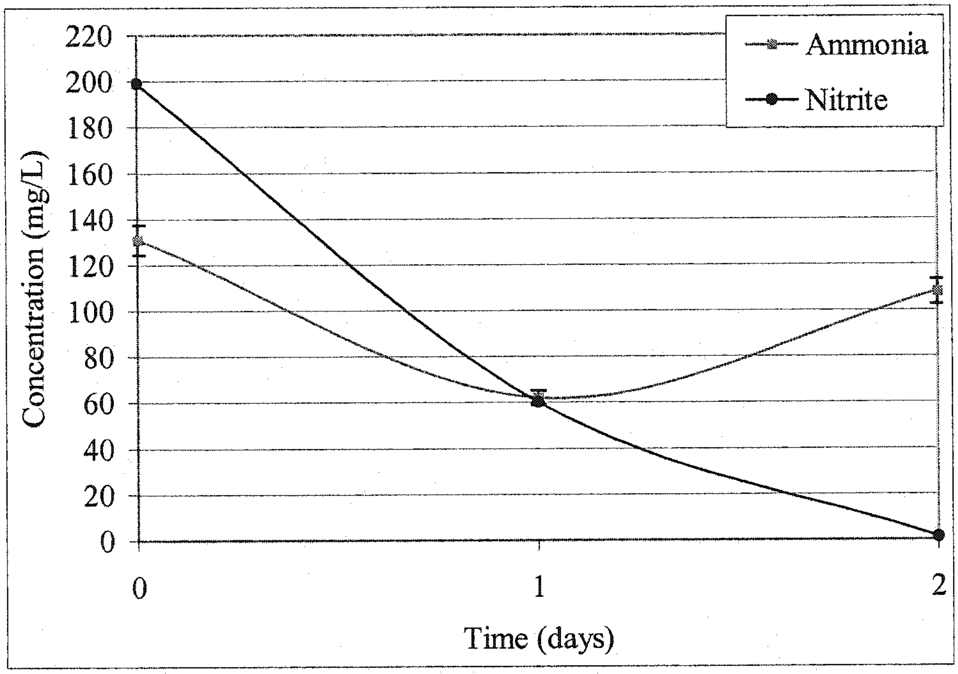


Figure 6.13: Variation of pH (pH = 6), ammonia and nitrite concentration

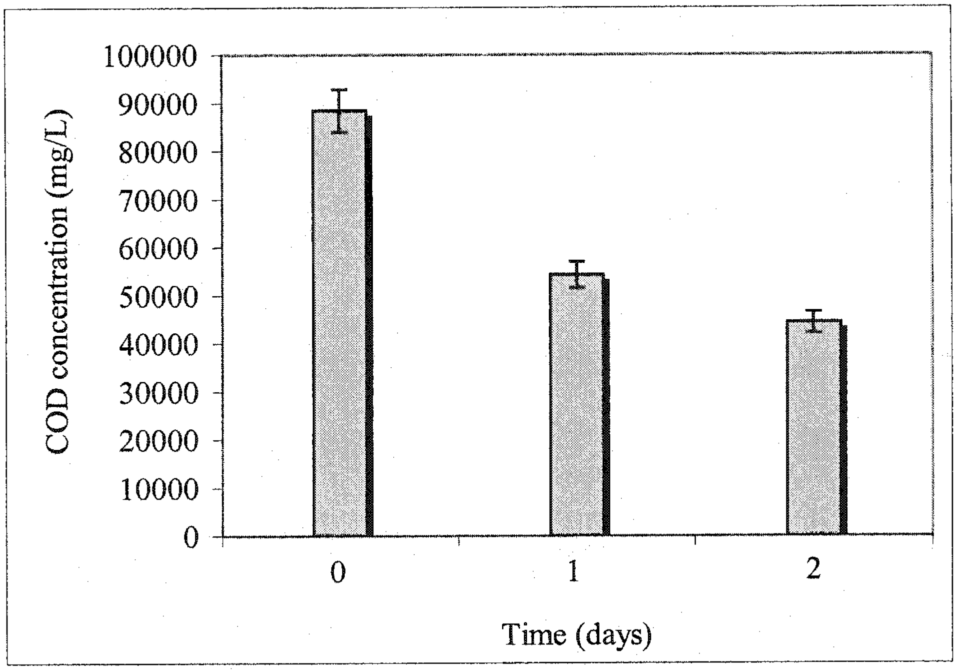


Figure 6.14: Variation of pH (pH = 6), COD concentration

6.2 Continuous flow experiment results

6.2.1 Volatile suspended solids (VSS)

After obtaining the important parameters from the batch experiments for the Anammox process, a continuous flow system was started with an anaerobic continuously stirred tank reactor. As mentioned in section 5.3.2, the Anammox population was enriched in the SBR in this study. A 2 L SBR was inoculated with 1 L biomass at the beginning. The defined media, glucose as well as nitrite were supplied to the SBR with a hydraulic retention time of 2 days. After stabilizing the system for several weeks, it was observed that the accumulated biomass remained in its granular shape. Then, the SBR was changed to the anaerobic continuously stirred tank reactor by being filled continuously. Samples were taken at that time ($t = 0$) and in every 2 weeks thereafter for total suspended solids and volatile suspended solids analysis.

The increase of total suspended solids (TSS) and volatile suspended solids at the end of the experiment (t =126 days) were about 8% and 5%, respectively. Figure 6.15 shows the TSS and VSS results. The increase of TSS and VSS was small, but the wash out of biomass from the effluent also influenced the results. However, the growth observed was significant knowing that the anaerobic ammonium oxidizing population could be enriched in the reactor with the supply of the medium.

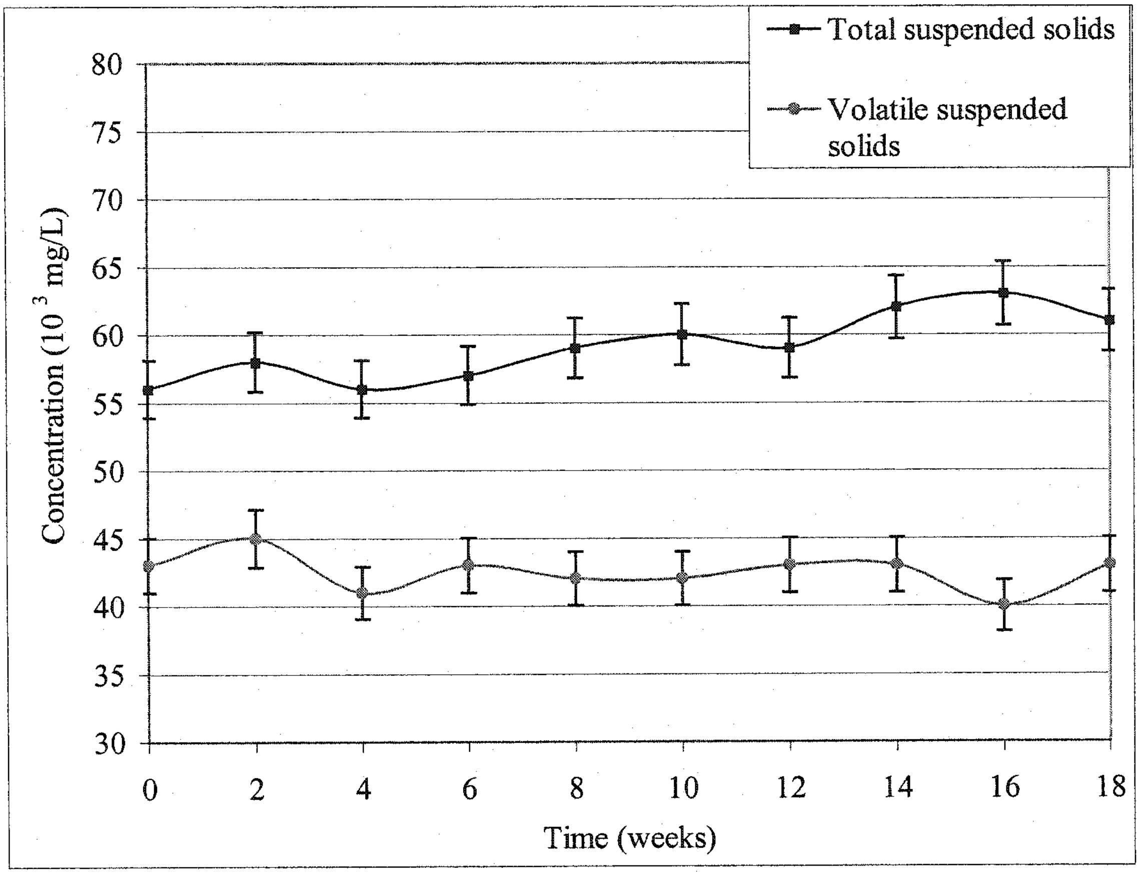


Figure 6.15: Results of total suspended solids and volatile suspended solids in the anaerobic continuously stirred tank reactor

6.2.2 Volatile fatty acids

As was indicated before, an ideal anaerobic process must first hydrolyze the substrate consisting of complex organic compounds to simple organics. After that, the simple organics such as sugars, amino acids are fermented to volatiles acids by acidogens bacteria (Mulligan, 2002). The volatile acids are then converted to acetate and H_2 gas and finally converted to methane gas by methanogens. Therefore, some volatile acids like acetic acid and propionate acid are significant indicator to the anaerobic process. Speece (1996) stated that propionate acid is a common intermediate in the metabolism of complex organics, and may account for 20 % of the flow that are eventually converted to methane. In order to investigate the effectiveness of the Anammox process in the anaerobic continuously stirred tank reactor, organic acids present in the medium were measured.

Figure 6.16 includes acetic, propionate and butyric acid concentration in the anaerobic continuously stirred tank reactor during 48 hour cycle. The increase of acetic, propionate and butyric acid at the end of this experiment was 0.05%, 0.04% and 0.06%, respectively. The amount of all acids kept increasing during the Anammox process. Moreover, it was noticed that the increase of the propionate acid was quite low and the low concentration of propionate is indicative of a well functioning anaerobic process (Speece, 1996).

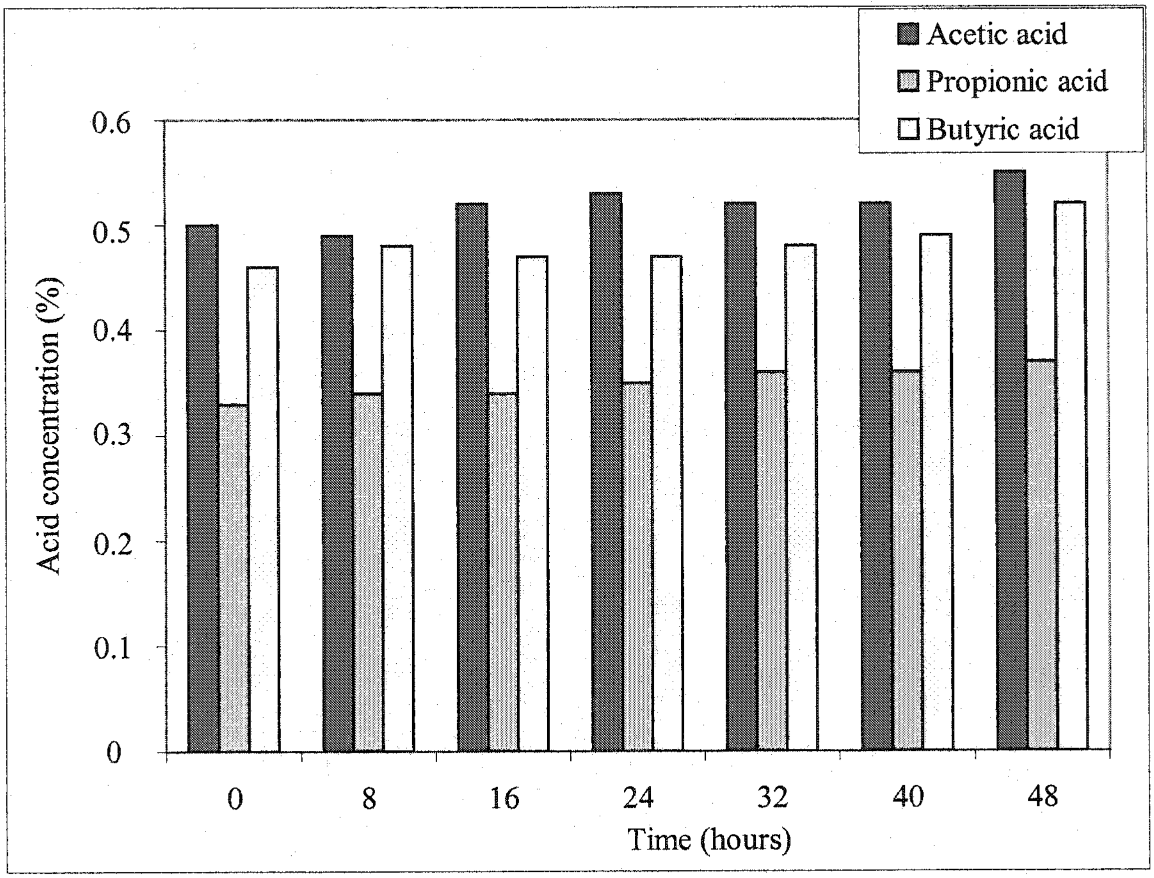


Figure 6.16: Organic acid concentration in the anaerobic continuously stirred tank reactor during one cycle of 48 hours

Afterwards, the hydraulic retention time in the anaerobic continuously stirred tank reactor was changed to 24 hours per cycle. Volatile fatty acids, principally acetic acid, were also produced in the medium. Figure 6.17 indicates acetic, propionate and butyric acid concentration in the SBR for 24 hours per cycle. The production of acetic, propionate and butyric acid at the end of this experiment was 0.02%, 0.02% and 0.04%, respectively. The concentration of all acids had increased in this experiment as well.

Therefore, these results support the evidence of well functioning anaerobic process. Besides, it is interesting to note that for both cases, the increase of volatile acids was small. According to Figure 6.16 and 6.17, it can be seen that the system is not overloading and the total nitrogen load could be increased.

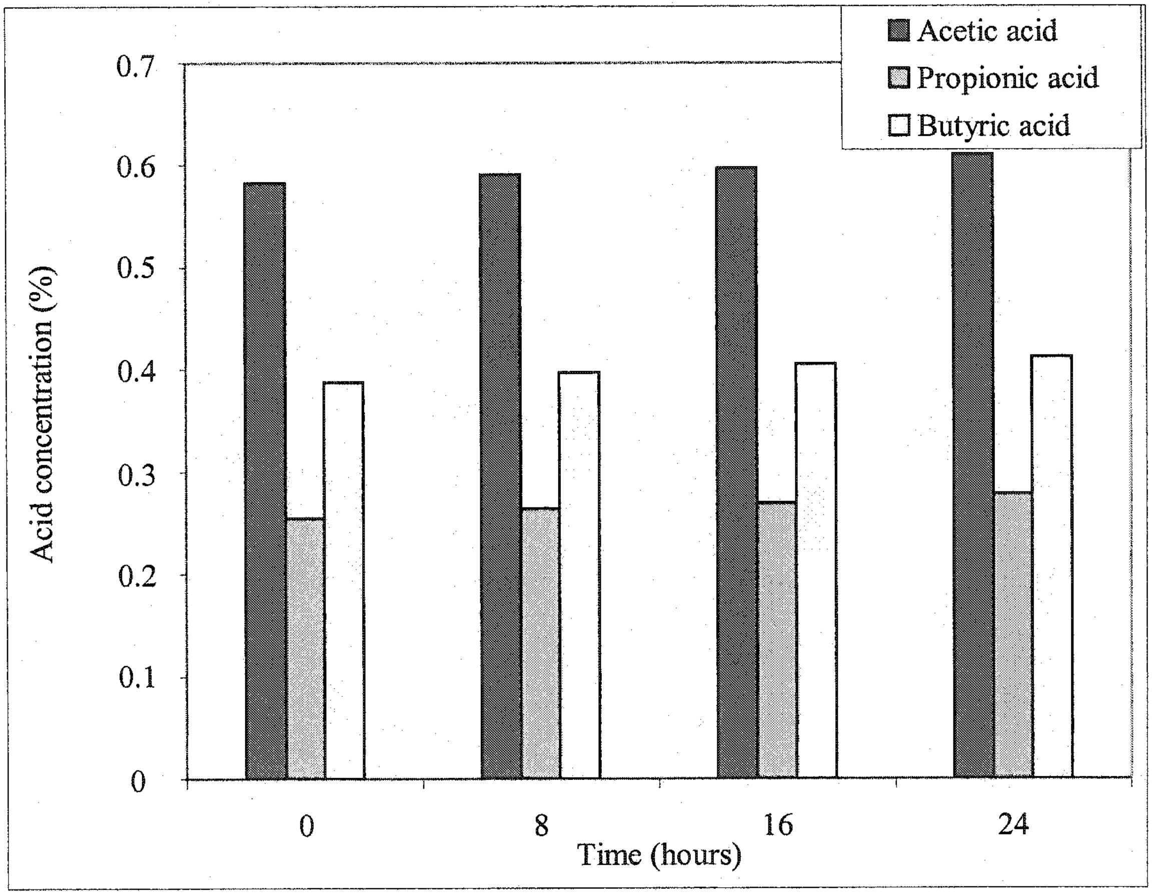


Figure 6.17: Organic acid concentration in the anaerobic continuously stirred tank reactor during one cycle of 24 hours

6.2.3 System with hydraulic retention time = 48 hours

The influence of oxygen on the Anammox process in the anaerobic continuously stirred tank reactor was also investigated in the continuous system. Initially, no ammonium ion was oxidized in the presence of 1 % of air in the anaerobic continuously stirred tank reactor. Hence, all the air was removed from the reactor by flushing the nitrogen gas. A dissolved oxygen probe monitored the level of oxygen and the process was controlled under strict anaerobic conditions. During the period of the experiment, it was found that the level of dissolved oxygen was equal to 0 %. In other words, it proves that the system was maintained under anaerobic conditions and there was no need to flush nitrogen gas into the anaerobic continuously stirred tank reactor for the remaining period. The ammonium ion and nitrite level at the influent and at the effluent were also tested. At $t = 30$ days, it was observed that the conversion of ammonium ion and nitrite occurred. After another month, at $t = 60$ days, the operation of the anaerobic continuously stirred tank reactor became more stable and samples were taken every 4 hours for analytical purposes.

Figure 6.18 includes COD, ammonia (NH_4^+), and nitrite (NO_2^-) concentration profiles for the hydraulic retention time of 48 hours. Nitrate production was relatively small and was not shown in the results. During this experimental period, after $t = 4$ hour, ammonium ion concentration started decreasing slowly. In contrast, the nitrite reduction after 4 hour was relatively fast. The maximum ammonium ion removal appeared at $t = 24$ hour, from its initial concentration of 226.5 mg NH_4^+–N /L to 96.3 mg NH_4^+–N /L. After that, ammonium ion level raised slightly again. This phenomenon could be due to the nitrite limitation in the system. As indicated in Figure 6.18, after *t* =28 hour, nitrite concentration dropped rapidly and was close to 0 mg NO_2^- –N /L after t = 40 hour. At the end of this experiment, the concentration of ammonium ion was 144.6 mg NH_4^+ –N /L and the removal efficiency was 36 %. On the other hand, nitrite conversion was fast and 99 % of nitrite was removed beyond *t* = 40 hour. The other main concern was the COD level in the system. The final COD concentration at *t* = 48 hour was 9,890 mg/L and the removal efficiency was about 77 %.

This can show that the Anammox process can have ammonium ion as well as nitrite removal coupled with COD reduction. Moreover, nitrate production at the end of this experiment was only 13 mg NO_3^- –N /L, which was very little. With the hydraulic retention time at 48 hours interval, however, the removal rate of ammonium ion is not satisfactory. As was observed, the maximum ammonia removal occurred at *t* =24 hour. Consequently, another hydraulic retention time of 24 hours was chosen to run the Anammox process in the anaerobic continuously stirred tank reactor.

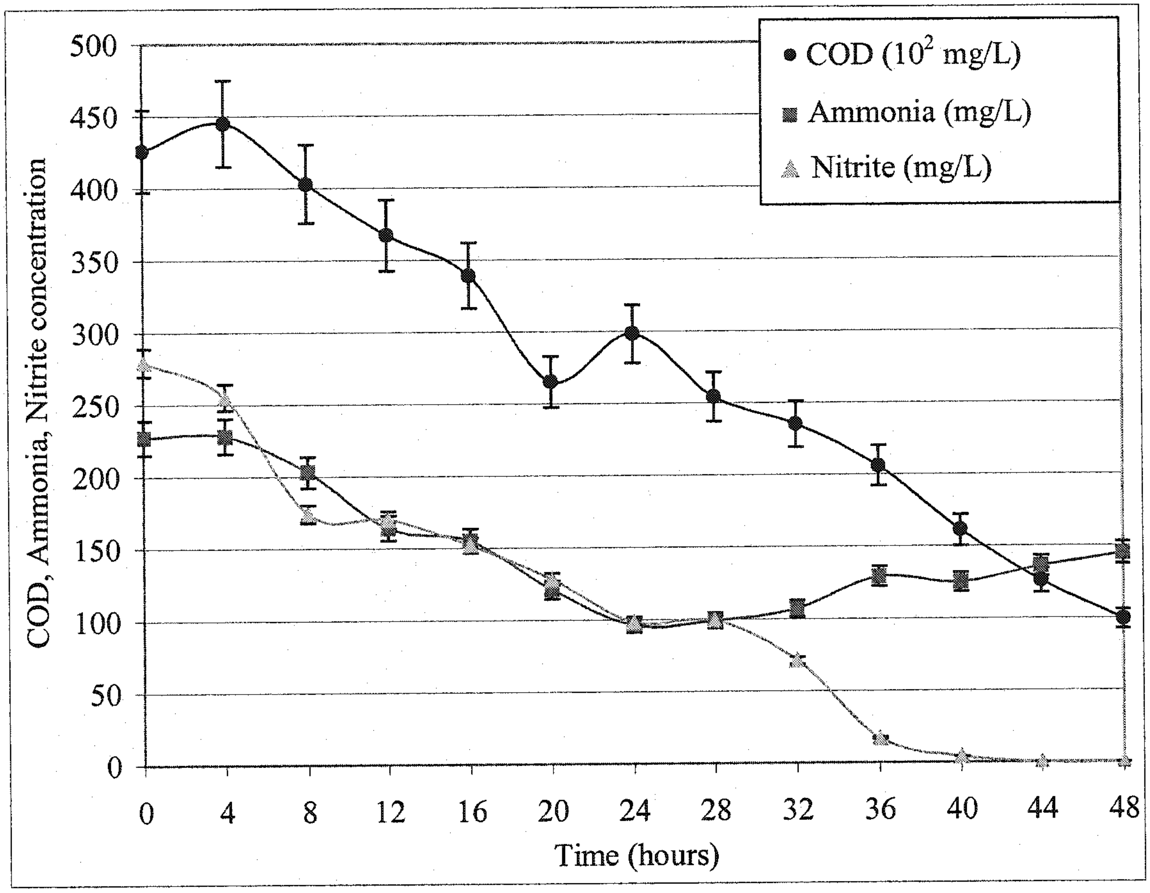


Figure 6.18: Concentration profiles in the anaerobic continuously stirred tank reactor during one cycle (HRT =48 hours)

6.2.4 System with hydraulic retention time = 24 hours

Since several weeks had to be taken for analyzing the samples previously obtained, the defined media, glucose, and ammonia were continually supplied to the anaerobic continuously stirred tank reactor so as to keep the biomass active. During this period, the anaerobic continuously stirred tank reactor was still strictly monitored under anoxic condition and the dissolved oxygen was maintained at 0 %. Figure 6.19 presents the concentration profile of COD, ammonia and nitrite in the system during one cycle (hydraulic retention time of 24 hours). In keeping with the experiment's consistency, the anaerobic continuously stirred tank reactor was stabilized for a month and nitrite was added after the month has elapsed. After 30 more days, it was noted that the conversion of ammonium and nitrite was stable, samples were then taken every 3 hours (from $t = 0$ to 24 hours).

From Figure 6.19, the level of ammonium ion in the anaerobic continuously stirred tank reactor decreased steadily after $t = 3$ hour. Beyond $t = 6$ hour, the ammonium ion concentration dropped from its initial concentration of 162.5 mg $NH_4^+ - N$ /L to final concentration of 70.8 mg $NH_4^+ - N$ /L. As expected, the final ammonium ion removal efficiency increased to 56 %. As shown in Figure 6.19, ammonium ion conversion was coupled with nitrite reduction. At the end of this experiment, nitrite concentration in the effluent was measured as 42.7 mg $NO_2^- - N$/L. Although the nitrite was not completely eliminated, 80 % of nitrite removal was achieved. The final result, on the other hand, indicated that only 50 % of COD removal could be reached. The main reason is because the retention time was shorten in this experiment.

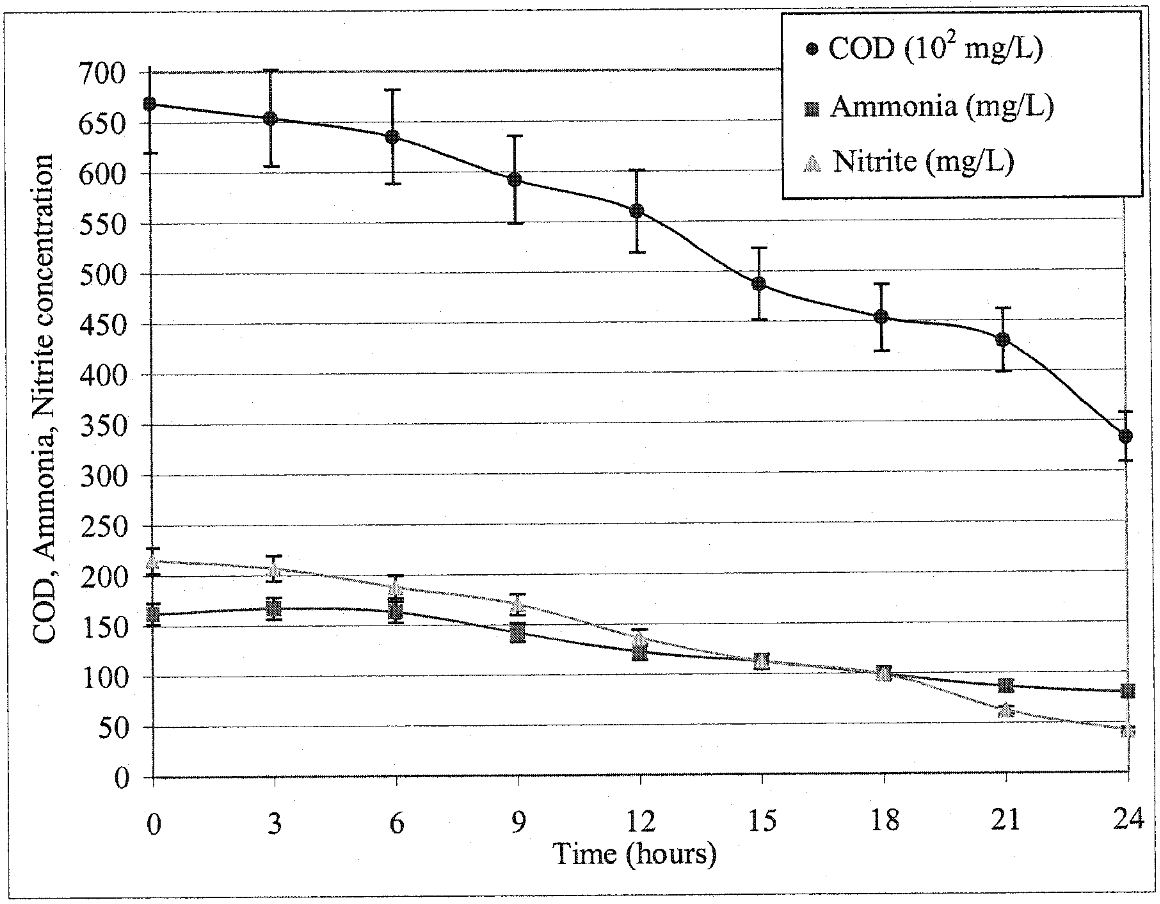


Figure 6.19: Concentration profiles in the anaerobic continuously stirred tank reactor during one cycle (HRT = 24 hours)

6.3 Summary

From this continuous flow experiment, it showed that the removal of ammonium ion was possible provided that nitrite was supplied to the system as an electron acceptor. The results of this study revealed some important points for anaerobic ammonium oxidation process:

1. The start up for the Anammox process is long; it takes few months in an effort to reach its stable operation. As mentioned in the batch results section, corrected COD : N ratio can stimulate the start up of the Anammox process. When it turns out that the enrichment in the reactor works well and ammonium oxidation becomes stable, the COD level in the influent can be lowered. In the study described here, the COD concentration in the influent was not adjusted in order to maintain consistency in the laboratory testing.

2. The existence of oxygen level is crucial to the Anammox process. No ammonium ion was oxidized in the presence of air, thus indicating that the Anammox activity in these enrichment cultures is only possible under strict anoxic conditions.

3. As indicated in the results, the hydraulic retention time is one of the key factors to the Anammox process. When the hydraulic retention time is longer

(HRT of 48 hours), higher COD removal and 99 % of nitrite reduction can be attained. However, the final ammonium removal efficiency is not adequate due to the nitrite limitation. This is in contrast to the observations made with different hydraulic retention time. When the hydraulic retention time is reduced to 24 hours per cycle, more than 50% of ammonium ion conversion is accomplished. Nevertheless, COD and nitrite removal efficiency decreases. According to the results in this study, ammonium ion conversion is also dependant on the nitrite level in the system. As presented previously, the level of nitrite after 24 hours is so low to have ammonium oxidation in the medium. One of the solutions is to supply extra nitrite to the system after 24 hours to promote the Anammox process. Nonetheless, it is essential to realize that the addition of nitrite must be controlled carefully since Anammox activity will be inhibited in the presence of high concentration of nitrite.

4. The difference of ammonia removal efficiency between this study and the Sharon – Anammox process presented in section 3.4.5.4 was 29%. This may be due to several reasons. First of all, Anammox reactors in the Department of Biotechnology at the Technical University of Delft, the Netherlands have been in operation for a long time. As a result, the organisms responsible for the Anammox process were all well grown in the reactors and the conversion rate proved to be more stable. Moreover, according to Van Dongen *et al.* (2001), the Sharon process operated without biomass retention, in other words, all nitrifying biomass will wind up in the Anammox reactor. Therefore, there was

no need for the biomass to adapt to the wastewater. The use of materials may be another factor. The synthetic wastewater utilized in the Sharon – Anammox process was composed differently than the defined media used in this research. For this reason, the growth of Anammox cells might have been influenced. There is no doubt that the Sharon – Anammox process proved to be a better alternative for the ammonia removal in wastewater when compared to the conventional nitrogen removal process. However, the results of this research demonstrate a few advantages over the Sharon – Anammox process. Without the preceding nitrification step, less energy (oxygen) is required. Additionally, the sludge production from the Anammox process is nearly negligible, and the Anammox process can be applied in the existing reactor type. It is possible to scale up the process directly to full-scale.

CHAPTER 7

CONCLUSIONS

The main purpose of this research is to evaluate the feasibility of anaerobic ammonium oxidation with a supply of extra nitrite to a biological process. This study has shown that removal of ammonium ion with the Anammox process is practicable when nitrite is applied directly to the biological wastewater treatment system. From the batch results of this study, it indicated that a COD to N ratio (150 : 1) gave greater potential to start up the process. From the previous research, the optimum range of pH for the Anammox process was between 7.0 and 8.5 (Mike *et al.*, 1998). Experiments with a series of the batch tests proved that decrease of pH to 6 did not negatively affect the process. During experiments with the defined media, the amount of nitrite addition was adjusted to be same concentration of ammonium ion since high nitrite concentration deteriorated the Anammox process. Furthermore, it was observed that temperature (30 – 35°C) was determined to be favorable for the system. The batch experiments showed that ammonium ion reduction usually occurred during the first 24 hours. However, it was noticed that the ammonium ion concentrations increased at the end of the experiment. It also illustrated that about 99% of nitrite removal could be achieved in the Anammox process with very little amount of nitrate production. Table 7.1 summarizes some of the results from the batch tests.

Table 7.1: Summary of the ammonium ion removal results from batch experiments

	Ammonia removal (%)	COD removal (%)	Nitrite removal (%)	Time interval (hours)
Case 1 **(COD : N = 75 : 1)**	29.9	32.6	90.1	0 – 24
	12.7	22.1	100	24 – 48
Case 2 **(COD : N = 150 : 1)**	26.3	33.3	93	0 – 24
	26.7	37.2	100	24 – 48
Case 3 **(COD : N= 75 : 1)** **Temperature = 35°C**	19.7	23.8	74.4	0 – 24
	10.9	36.1	98.9	24 – 48
Case 4 **(COD : N = 150 : 1)** **Temperature = 35°C**	55.6	50.9	66	0 – 24
	49.4	56.4	98.6	24 – 48
Case 5 **(COD : N = 150 : 1)** **Temperature = 35°C** **pH = 6**	53.1	38.7	70	0 – 24
	17.7	49.9	99.6	24 – 48

Applying the parameters that were attained in the batch experiments, the Anammox reactor inoculated with the microorganisms was started up for the continuous flow system research. From the batch tests, it was known that the presence of oxygen plays a significant role on the Anammox process. In order to have a successful Anammox process, the oxygen level in the anaerobic continuously stirred tank reactor must be kept at 0%. The increase of volatile suspended solids at the end of this experiment was 0.134 g/L. The growth observed was significant knowing that the biomass could be enriched with the medium and nitrite addition condition. However, the growth was very small and it revealed a clear advantage of this process with a low sludge production. Table 7.2

reveals the summary of the results for the anaerobic continuously stirred tank reactor. With hydraulic retention time of 48 hours, higher COD removal was achieved but the ammonium ion removal efficiency was decreased. On the contrary, lower the hydraulic retention time to 24 hours, more ammonium ion could be removed.

Table 7.2: Summary of the results for the anaerobic continuously stirred tank reactor

	Case 1 HRT = 24 hours	**Case 2** HRT = 48 hours
Influent COD (mg/L)	66000	42600
Effluent COD (mg/L)	32100	9890
COD removal (%)	**50.2**	**76.8**
Influent NH_4^+ (mg/L)	162.46	226.54
Effluent NH_4^+ (mg/L)	70.88	144.58
NH_4^+ removal (%)	**56.4**	**36.2**
Influent NO_2^- (mg/L)	215.28	278.90
Effluent NO_2^- (mg/L)	42.70	0
NO_2^- removal (%)	**80.2**	**100**

7.1 Recommendations

In fact, a number of technical and economical problems have remained, concerning the practicalities of applying the nitrite directly on an industrial scale for the Anammox process. Long start up of the Anammox reactor is one of the most critical aspects found in this research. Moreover, ammonium ion removal and COD reduction are between 55 and 60 %, which is considered not sufficient enough.

Nevertheless, these problems may be overcame by a two – step procedure in which acetogenesis and methanogenesis are performed as separate processes or a multi-stage system like the multiplate reactor. Since the pH preference of acid and methane-forming bacteria are different, separate reactors can be designed for each step for optimal performance. The formation of the volatile acids would take place in the first reactor and the methane production could occur in the second one. This appears to increase the overall treatment efficiency. Because of the additional reactor, the capital cost is raised. Therefore, the profitable application is a matter of cost analysis. Finally, it is hoped that this study will stimulate further investigation in this topic.

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