University of Stavanger

Faculty of Science and Technology

MASTER'S THESIS

Study program/ Specialization: Environmental Technology/ Offshore	Spring semester, 2016 Open access
Writer: RAOELIARITIANA Jany Olivia	…… (Writer's signature)

Faculty supervisor: Krista Kaster and Andrea Bagi

Thesis title:

Methods for determining the activity of nitrifying bacteria in wastewater samples

Credits (ECTS): 30

Key words: - Nitrification - Wastewater, - Temperature, - Nitrifying bacteria - Growth - DGGE	Pages: 56 + enclosure CD Stavanger, 15 June 2016

Methods for determining the activity of nitrifying bacteria in wastewater samples

Faculty of Science and Technology

Department of Mathematics and Natural Science

Master Degree Program on Environmental Technology

University of Stavanger, Norway

RAOELIARITIANA Jany Olivia

Acknowledgements:

This research was established at the Faculty of Science and Technology at the University of Stavanger in order to complete a Masters' degree in Environmental technology completed.

First and foremost, I would like to express my profound gratitude to Professor Torleiv Bilstad for giving me the opportunity to study at the University of Stavanger.

I am very grateful to my supervisor Krista Kaster for her support, guidance, patience and motivation throughout the completion of this work. Her contributions of time and ideas are greatly valued. Her advices were not only essential to this work but also helped me gain insights into the workings of academic research in general.

I would like to thank also Andrea Bagi for her help during my laboratory experience.

Above all, I would like to thank to my beloved family for their love and support in my entire life. They are always there for me no matter what, no matter when.

Abstract:

The present study investigated the feasibility of nitrification in wastewater and the community structure of nitrifying bacteria at two different temperatures, 10 and 25°C. The experiment was conducted in sequencing batch reactor (SBR). During this experiment, ammonia, nitrite and nitrate analysis were achieved to determine the potential nitrification. The community diversity of nitrifiers was explored by using a culture-based method: most probable number (MPN) and a molecular based method denaturing gradient gel electrophoresis (DGGE). We observed that nitrification occurred at both tested temperatures. The result of nitrification showed that the ammonia, nitrite and nitrate concentration were lower at 10°C compared to those incubated at 25°C. The nitrification rate showed also that there was a slight difference between the wastewater incubated at 10°C and 25°C, as the nitrification appeared to be faster at 25°C than at 10°C. The experimental results indicated that temperature is an important factor affecting the nitrification process. According to the MPN results, the abundance of the nitrifiers increased throughout the experiment. The MPN results also showed that the concentration of nitrifiers was higher at 25°C than at 10°C. Furthermore, the band patterns observed in the DGGE analysis revealed a shift in community composition over time and also showed that the day 60 (final) communities were different at 10°C compared to 25°C. The higher number of bands indicated that the bacterial diversity was higher at 25°C than at 10°C. To summarize this study, an effect of change in temperature has been observed during the nitrification process, which affect at the same time the change in bacterial diversity of nitrifying bacteria.

Key words: nitrification, wastewater, temperature, nitrifying bacteria and DGGE.

Table of contents:

Lists of figures:

Lists of tables:

Abbreviations:

$(NH_4)_2SO_4$: ammonium sulfate

AMO: Ammonia monooxygenase

AnAOB: anaerobic ammonia oxidizing bacteria

AOA: ammonia oxidizing archea

AOB: Ammonia Oxidizing Bacteria

ATP: Adenosine tri-phosphate

$CaCl_2.2H_20$: Calcium chloride dehydrate

CFU: Colony forming unit

$CoCl_2.6H_2O$: Cobalt chloride hexahydrate

COD: Chemical oxygen demand

$CuCl_2.2H_2O$: Copper chloride dihydrate

$CuSO_45H_2O$: Copper sulfate pentahydrate

DGGE: denaturing gradient gel electrophoresis

dH_2O: distilled water

DNA: deoxyribonucleic acid

DO: dissolved oxygen

dsDNA: double strand DNA

EDTA: ethylene diamine tetra acetic acid

$FeCl_2.4H_2O$: Iron chloride tetrahydrate

$FeSO_47H_2O$: iron sulfate heptahydrate

FISH: fluorescence in situ hybridization

H_3BO_3: Boric acid

HAO: hydroxylamine oxidoreductase

HCl: hydrogen chlorid

HNO_2: nitrous acid

HRT: Hydraulic Retention Time

K_2HPO_4: di-potassium hydrogen phosphate

KH_2PO_4: potassium dihydrogen phosphate

$K_{S,O2}$: half saturation concentration of oxygen

$MgCl_2.6H_2O$: magnesium chloride hexahydrate

$MgSO_4.7H_2O$: magnesium sulfate heptahydrate

$MnCl_2$: Manganese chloride

$MnCl_2.4H_2O$: Manganese chloride tetrahydrate

MPN: most probable number

NA: non applicable

N-1-NED: *N*- (1-naphthyl)-ethylenediamine dihydrochloride

N_2: nitrogen gas

N_20: nitrous oxide

$NaMoO_4.2H_2O$

NaOH: sodium hydroxid

NH_3: ammonia

NH_4^+: ammonium

NH_4Cl: ammonium chloride

$NiSO_4.6H_2O$

NO_2: nitrogen dioxide

NO_2^-: nitrite

NO_3^-: nitrate

NOB: Nitrite Oxidizing Bacteria

NOR: Nitrite Oxidoreductase

PCR: Polymerase chain reaction

qPCR: Real time quantitative PCR

RNA: ribonucleic acid

rRNA: ribosomal RNA

SD: Standard deviation

SRB: sequencing batch reactor

SRT: Solid Retention Time

T-RFLP: Terminal restriction fragment length polymorphism

T: temperature

UV: ultra-violet

V: volt

VSS: volatile suspended solids

WWT: wastewater treatment

$ZnCl_2$: Zinc chloride

$ZnSO_47H_2O$: Zinc sulfate heptahydrate

I. INTRODUCTION:

Nitrification is an important process in the nitrogen cycling and a vital component in the activated sludge wastewater treatment plant (WWTPs) (Juretschko et al,.1998). It plays an important role in such plants by reducing the oxygen demand of ammonia and organic nitrogen. It is the opening step of complete removal of nitrogen via denitrification in wastewater (Hall and Murphy, 1980).

A typically harmful contaminant in wastewater is ammonia. Ammonia in wastewater comes from several sources including agricultural and industrial waste, surface runoff and leachates (EPA, 1993). Ammonia causes pollution to the environment. Its accumulation in wastewater may have adverse effects to the human health, aquatic life, and the environment. It is necessary to reduce ammonia content of sewage because of its toxicity and side effect to the environment (Purkhold et al., 2000). It underlies a serious problem in aquatic ecosystem, which causes eutrophication and blue baby syndrome in drinking water consumption (Ge, Jiang, & Wei, 2015). High concentrations of ammonia in wastewater may be toxic to fish and others species and can lead to eutrophication and algal bloom (Forgie, 1988; Welander, Henrysson, & Welander, 1998). Other destructive impacts resulting from the high concentration of ammonia in wastewater include public health (water consumption), dissolved oxygen depletion, reduction of the effectiveness of chlorine disinfection and reduction in recycling the wastewater (De Renzo, 1978). As a result, EPA has limited the suitable ammonium concentration discharge in wastewater to <10 mg/l (EPA, 1993; Welander, Henrysson, & Welander, 1997). Seeing these effects, nitrogen in wastewater must be removed by biological treatment: nitrification and denitrification.

The concentration of ammonia in wastewater depends on several parameters like temperature, dissolved oxygen, free ammonia, pH, solid retention time (SRT), salinity and hydraulic retention time (HRT) (Quinlan, 1984). Temperature plays an important role during nitrification because it affects the metabolism and the growth rate of nitrifying bacteria. Since temperature cannot be controlled manually, the effect of temperature in nitrification lets engineers to modify and to provide reliable and efficient level of treatment under less than optimal conditions.

The bacteria responsible for nitrification have a slow growth rate and sensitivity to toxic shocks and changes in operational and environmental conditions (Marisili-Libelli and Giovannini, 1997). To achieve a complete nitrification, an SRT between 3 to 4 days is required in an activated sludge and if nitrification is to be avoided, the SRT should be smaller (Grady et al, 1999). It is also accepted that the SRT required for complete nitrification increased with decreasing temperature and dissolved oxygen (e.g. Wild et al., 1971; McClintock et al., 1993; Ydestbo et al., 2000). Nitrifying bacteria are difficult to cultivate and the culture-dependent enumeration techniques result in an underestimation of the

number and diversity (Juretschko et al., 1998). Due to the difficulties associated with quantifying nitrifiers via cultivation techniques, culture-independent technique has been pursued. These tools consist of detection and quantification of the target molecule in specific microbial population. Together with culture based method and molecular tools, these techniques show an opportunity to define and to improve the reproducible method to quantify nitrifiers.

This study is based on the comparison of methods used to determine nitrification in wastewater and nitrifying bacteria via traditional methods and molecular techniques. This study investigated the effect of temperature on nitrification rate. It also looked at the changes in the composition of the evolving nitrifying bacterial community to investigate if a low temperature affected the structure of the microbial community compared to samples incubated at higher temperature. This was performed by incubating wastewater samples at different temperatures over 60 day period and by looking at changes in the microbial community using denaturant gradient gel electrophoresis (DGGE) and at the changes in nitrifier abundance by using Most Probable Number (MPN) method. The entire length of the study period has been 70 days (01 March to 09 June).

II. Background:

2.1. Nitrogen cycling:

The ecosystem encompasses nitrogen in various forms. And it is the most abundant element found in wastewater and constitutes 78% part of the atmosphere. Its presence in wastewater can damage the associated ecosystem. Therefore, several methods have been designed for nitrogen removal from wastewater (EPA, 1993).

The nitrogen cycle is a complex biochemical process. The biochemical cycle of nitrogen refers to the transformation to its various forms of oxidation: ammonia, nitrate, nitrite, nitrogen dioxide, nitrous oxide, nitric oxide, and nitrogen gas in the environment (Arp, Sayavedra-Soto, & Hommes, 2002). The different steps of nitrogen cycle include nitrogen fixation, ammonification, nitrification, assimilation, denitrification and decomposition. Specific bacteria carry out the transformation of nitrogen. These reactions occur in nature, and bacteria transformed organic matter to ammonia. The first step of nitrogen cycle is nitrogen fixation. The organic nitrogen is transformed into ammonia. Secondly, the ammonia is oxidized into nitrate where nitrite is the intermediate step. This process is called nitrification and performed by nitrifying bacteria. After nitrification, plants and animals will absorb nitrate, this process is called assimilation. During assimilation, nitrate is converted into organic nitrogen. After assimilation, specialized decomposing bacteria will convert the organic nitrogen into ammonia. This process is called ammonification. After that, nitrate will be reduced to nitrogen gas (Ragsdale, 2013).

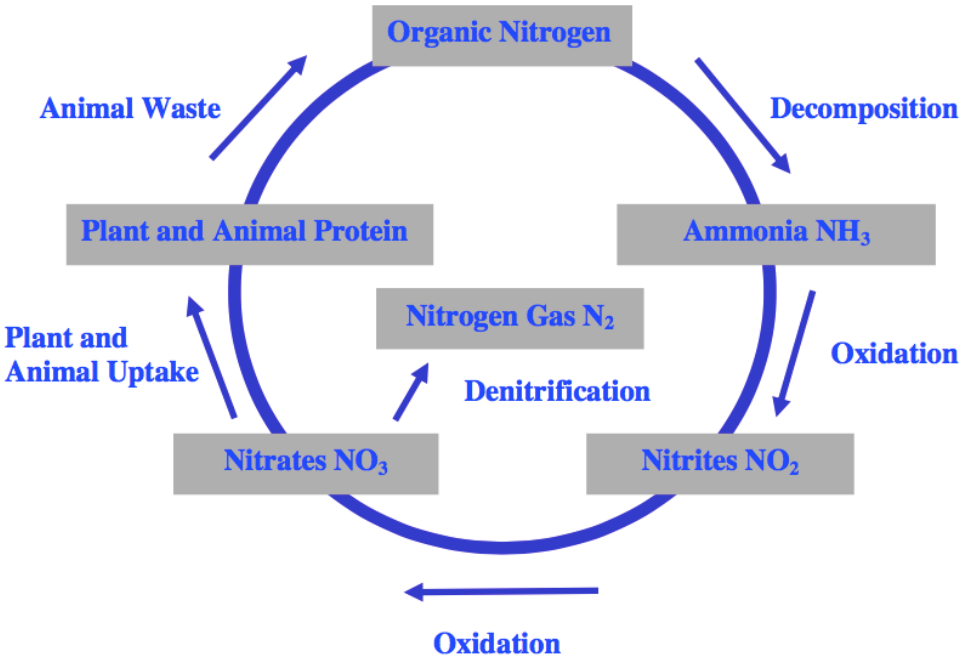

Figure 1: Overview of nitrogen cycle copied from (Ragsdale, 2013)

2.2. Nitrification

The biological oxidation of ammonia occur in two steps and is carried out by chemoautotrophic groups of bacteria (Prosser, 1989). Ammonia-oxidizing Bacteria (AOB) and Ammonia-oxidizing Archaea (AOA) perform the oxidation of ammonia, where AOA appear to dominate the environment (Könneke et al., 2005). *Thaumarchaeota,* an AOA species could play an important role during the ammonia oxidation. These bacteria use ammonia and nitrite as the electron donor and carbon dioxide for carbon source (Rittmann & McCarty, 2012). During the first step of nitrification, AOA and AOB oxidize ammonia to nitrite converted by ammonia monooxygenase (AMO) yielding hydroxylamine (Bothe, Jost, Schloter, Ward, & Witzel, 2000) following this equation:

$$NH_4^+ + O_2 + H^+ + 2e^- \rightarrow NH_2OH + H_2O$$

And after that, the hydroxylamine is oxidized to nitrite by AOB species by the enzymes hydroxylamine oxidoreductase (HAO) via the below reaction:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

The second step of nitrification yields nitrate and carry out by NOB. This process is catalyzed by the enzyme nitrite oxidoreductase (NOR) (Gray, 1989), following this equation :

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$$

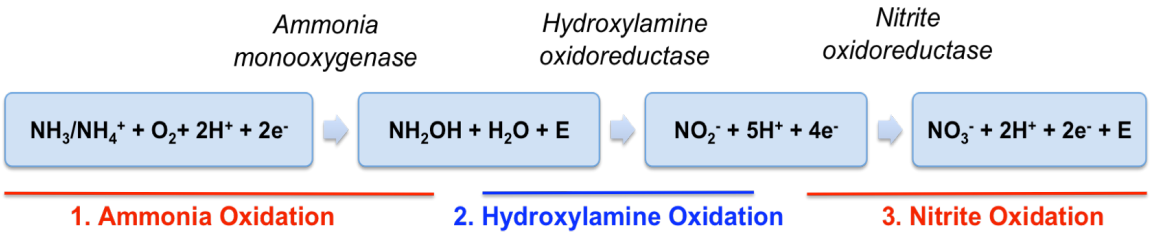

Figure 2: Overall diagram of the nitrification process. Ammonia monooxygenase, hydroxylamine oxidoreductase and nitrite reductase are the enzymes involved in these reactions. E represents energy released from this process. Copied from Whalen (2010)

2.3. Microorganisms involved in the nitrogen cycle:

In the nitrogen cycle microbial communities represent a fundamental part since they are responsible for the transformation and energy fluxes. Four groups of microorganisms are involved: Ammonium Oxidizing Bacteria (AerAOB), Ammonium Oxidizing Archaea (AOA), Nitrite Oxidizing Bacteria (NOB) and Anoxic Ammonium Oxidizing Bacteria (AnAOB).

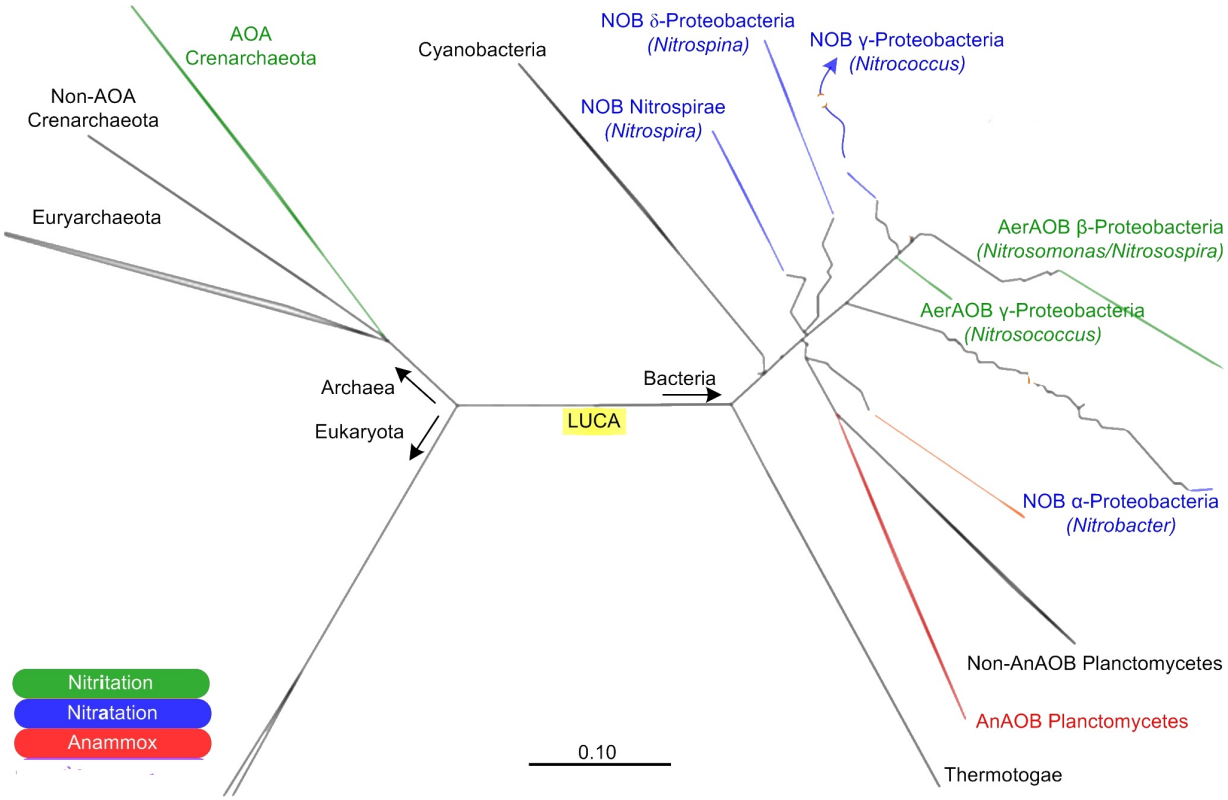

Figure 3: Phylogenetic tree based on 16S rRNA of nitrifying bacteria. Nitritation process microorganisms: AOA and AerAOB (in Green), nitratation microorganisms: NOB (in blue) and AnAOB (in red). Copied from Vlaeminck et al (2011)

Table 1: Summary of environmental factors for N oxidizing microbes (Anthonisen, Loehr, Prakasam, & Srinath, 1976; Grüntzig, Stres, del Rio, & Tiedje, 2002; Hatzenpichler et al., 2008; Könneke et al., 2005; Martens-Habbena, Berube, Urakawa, José, & Stahl, 2009; Prosser, 1989)

Parameters	AerAOB	AOA	NOB	AnAOB
Temperature (°C)	25-30	18-74	26-46	12-43
pH	7.2-8.2	5.5-7.2	7.6-8.2	6.7-9
Grow rate ($days^{-1}$)	0.48	0.78	0.264-0.33	0.069
Oxygen affinity constant (Ks) (mg O_2/l)	0.6	0.124	2.2.	NA
DO (mg O_2/l)	NA	NA	<1	≥ 0.03
Free ammonia (mg N/l)	8-120	43	0.08-0.82	NA
NO_2 (mg N/l)	NA	NA	NA	100-350
HNO_2	0.2-2.8	0.06-0.8	0.06-0.8	NA

2.3.1. AOB:

AOB carry out the oxidation of NH_3 to NO_2^-. They are obligate chemolithoautotrophs and have the ability to satisfy their requirement for growth via carbon source (Wood, 1986). According to many authors, *Nitrosomonas europaea* is the model of bacteria for ammonia oxidation, nevertheless other ammonia-oxidizers are also important (Wagner et al., 2002). Most of AOB belong to the β-subclass of Proteobacteria, although some of AOB belong to γ-subclass of Proteobacteria.

Five genera of AOB have been identified based on their structural properties: *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosovibrio* and *Nitrosolobus* (Madigan, Martinko, Parker, & Brock, 1997) but this has been reduced into three genera due to 16S rRNA analysis (De Beer & Schramm, 1998). So, *Nitrosococcus mobilis* belong to the genera of *Nitrosomonas*, while *Nitrosolobus, Nitrosospira* and *Nitrosovibrio* are all belong to the genera of *Nitrosospira.* The remaining AOB are affiliated to the genera *Nitrosococcus* (Wagner et al., 2002). *Nitrosomonas* and *Nitrospira* belong to β-Proteobacteria and *Nitrosococcus* belongs to γ-Proteobacteria. *Nitrosomonas* and *Nitrosospira* are found in diverse ecosystems, while *Nitrosococcus* are found in marine ecosystem. A spherical cell shape characterizes *Nitrosococcus*, while *Nitrosomonas* has straight rods cell and *Nitrospira* a tightly spiral shape. *Nitrosomonas europaea*, a species of AOB performs the first step of nitrification. AMO catalyzed the oxidation of ammonia to hydroxylamine, the intermediate product. Then, the oxidation of hydroxylamine to nitrite is catalyzed by HAO (Chang, Hyman, & Williamson, 2002; Keener & Arp, 1993; McCarty, 1999; Rasche, Hicks, Hyman, & Arp, 1990). The entire step of ammonia oxidation yields four electrons. Two electrons are transferred to AMO to maintain the rate of ammonia oxidation and to activate oxygen. And the last two electrons are used for the biosynthesis and ATP generation (Arp et al., 2002; McCarty, 1999; Wood, 1986). The equation of ammonia oxidation performed by AOB is:

AMO: $NH_3 + O_2 + 2H^+ + 2e^- + NH_2OH + H_2O$

HAO: $NH_2OH + H_2ONO_2^- + 5H^+ + 4e^-$

TERMINAL OXIDASE: $0.5\ O_2 + 2H^+ + 2e^-\ H_2O$

Furthermore, AMO is able to oxidize a wide range of non-growth substrates like aromatic hydrocarbon, aliphatic hydrocarbon and chlorinated compounds (McCarty, 1999; Wood, 1986). More than 40 compounds have been demonstrated to be non-growth substrates for AOB and inhibit ammonia-oxidation.

2.3.2. AOA:

The AOA are categorized to be prokaryotes as they are considered to have single-celled

organisms without nuclear membranes. This domain is classified into two subdivisions *Crenarchaeota*, which are predominantly hyperthermophiles and *Euryarchaeota*, which are methanogens and extreme halophiles (Woese*, et al.*, 1990). Both of them are usually found in extreme environments (Brown & Doolittle, 1997) and might play an important role in the biogeochemical cycles of the oceans (Ouverney & Fuhrman, 2000). It was assumed that only AOB is the only able to oxidize ammonia (Purkhold et al., 2000). However, some researchers have been discovered diverse groups of Archea, the *Crenarchaeota* groups able to oxidize ammonia to nitrite (Brochier-Armanet, Boussau, Gribaldo, & Forterre, 2008). AOA have been found to dominate the marine environment, especially to the deeper of ocean where for example *Nitrosopumilus maritimus* is the species of AOA isolated from a marine environment (Könneke et al., 2005) and able to grow at low concentration of ammonia. They have the capacity to scavenge ammonia located in such environment (Mincer et al., 2007). *Nitrosopumilus maritimus* belongs to the group I.1a Crenarchaeota (Fig3). The groups I.1a are found in the water column and sediment whereas the groups I.1b: *Nitrososphaera gargensis* and *Nitrososphaera viennensis*, are found in the soil and sediment (Hatzenpichler et al., 2008). Since AOA is recently discovered, they are not a great number of isolated species unlike AOB. To date, *Crenarchaeum symbiosum,* the Archea that live in symbiosis with the marine sponge *Axinella Mexicana* has been stated to be potentially autotroph and harbor the genes to encode AMO (Preston, Wu, Molinski, & DeLong, 1996). Thus, with their autotrophic metabolism, these species are capable to use reduced nitrogen as energy source (Schleper & Nicol, 2010). Nevertheless, several studies show that AOA are outnumber AOB in marine ecosystem (Erguder, Boon, Wittebolle, Marzorati, & Verstraete, 2009). *Nitrosopumilus maritimus* can grow with low concentration of ammonia 10nM (Martens-Habbena et al., 2009), which is 100 times lower than described to cultivate AOB. Therefore, in oligotrophic environment, these organisms can thrive despite of limited nutrients (Siripong & Rittmann, 2007). However, both AOA and AOB have the similar growth rate (Könneke et al., 2005).

2.3.3. NOB:

NOB are gram negative eubacteria. They are obligate chemolithoautotrophs meaning that they use nitrite as source of energy and CO_2 as the carbon source (Spieck & Bock, 2005). They have diverse forms: rods, spiral and cocci. The classification of NOB is based on the cell shape, where four genera of NOB have been identified: *Nitrococcus, Nitrobacter, Nitrospina* and *Nitrospira* (Belser, 1979; Bock et al., 1992; Juretschko et al., 1998). *Nitrobacter* is characterized as pleomorphic short rods with an intracytoplasmic membrane. *Nitrococcus,* is coccoid characterized with a tubular intracytoplasmic membrane. *Nitrospina*, are long rods without tubular intracytoplasmic membrane. A spiral shape and the absence of intracytoplasmic membranes characterize the *Nitrospira* genus (Spieck

& Bock, 2005). Unlike AOB and AOA, which are limited to two class of Proteobacteria, NOB are more dispersed. *Nitrobacter* belongs to α-Proteobacteria, *Nitrococcus* belongs to γ-Proteobacteria, *Nitrospina* and *Nitrospira* are affiliated to δ-Proteobacteria but it was demonstrated that *Nitrospira* belongs to the class of *Nitrospirae* (Spieck & Bock, 2005). Mobbary (1996) assumed that *Nitrobacter* is the best investigated NOB in most natural habitats and wastewater, it has the ability to tolerate the changes in environmental conditions. Several studies have shown that *Nitrospira* are dominant in wastewater compared to *Nitrobacter* (Burrell, Keller, & Blackall, 1999; De Beer & Schramm, 1998; Hovanec, Taylor, Blakis, & Delong, 1998). According this, it has been suggested that *Nistrospira* plays a more important role during nitrite oxidation than *Nitrobacter.* The predominance of *Nitrospira* compared to *Nitrobacter* in WWT is reflected in their survival strategy. According to the data obtained from fluorescence in situ hybridization (FISH), *Nitrospira* is categorized as K-strategists, and adapted at low concentration of nitrite and oxygen. However, *Nitrobacter* have been found to be r-strategists and have a low affinity for nitrite and oxygen (De Beer & Schramm, 1998). K-strategists are adapted more to nutrient limited environment and have high affinities for substrates. In contrast, r-strategists are fast growing and have low substrate affinities (Andrews & Harris, 1986). Since the concentration of nitrite in WWT is low, *Nitrospira* may out compete *Nitrobacter* (Wagner et al., 2002). Nitrifying organisms can survive and adapt longs periods of dryness and starvation even though they do not form endospore (Spieck & Bock, 2005). The table bellow summarized the different that exist between the four genera of NOB (table2).

Table 2: Differentiation of the four genera of nitrite-oxidizing bacteria (Spieck & Bock, 2005)

Characteristic	Nitrobacter	Nitrococcus	Nitrospina	Nitrospira
Phylogenetic position	α -Proteobacteria	γ -Proteobacteria	δ-Proteobacteria	Nitrospirae
Morphology	Pleomorphic short rods	Coccoid cells	Straight rods	Curved rods to spira
Intracytoplasmic membrane	Polar cap	Tubular	Missing	Missing
Motility	+	+	-	-
Location of the nitrite oxidizing system on membranes	Cytoplasmic	Cytoplasmic	Periplasmic	Periplasmic

2.4. Importance of nitrification in wastewater:

Access to clean water is essential for life. Nitrogen usually constitutes 40-50% of the impurities in wastewater. Nitrogen in wastewater is present in various forms: ammonia, organic nitrogen and urea. In wastewater treatment, the organic nitrogen is converted into ammonium. When released in the effluent, it will enrich the receiving water and can lead to eutrophication and bloom algal. Regarding these effects, nitrification is designed to remove inorganic nitrogen from wastewater. This is aerobic process, AOB, AOA and NOB carry out the reactions, and they required aeration for their growth and activity and yielding nitrate. Nitrate in wastewater should be removed during anoxic treatment via denitrification. In this process, nitrate is reduced to nitrogen gas, which is released into the atmosphere. Most research focus on the optimization of nitrification in wastewater treatment, especially of the nitrifying organisms and the factors that may inhibit the nitrification (Kuenen, 2008).

2.5. The microbial diversity in Wastewater:

The microbiology content of wastewater is variable. There may be 10^9 bacteria per gram of sewage. On an incredibly scale, wastewater contains heterogeneous mixture of organisms (Seviour & Blackall, 2012). The microorganisms in wastewater are derived from natural environment, pollution industrial and agricultural and human beings. They can be viruses, bacteria, fungi, bacteriophage, fungi and algae. The bacteria alone can show a diversity of metabolic characteristics like autotroph, heterotroph, aerobic, anaerobic, thermophile, and psychrophilic bacteria. The study of microbial diversity and the ecology of microorganisms in wastewater treatment are very important because the strategy of these systems is based on fundamentally microbiology (Wagner et al., 2002). The prediction of microbial diversity is intricate, based on experiment and mathematic modeling (Curtis & Sloan, 2006). For example, 'functional redundancy' describes a situation in which some species performs the same biochemical or metabolic function inside of community in order to prevent environmental failure. This has been observed in nitrifying species in wastewater (Rowan et al., 2003). The study of community dynamics will be the next level for the investigation of bacterial communities in wastewater treatment. It can be supported with thorough understanding the chemical, physical and biological of the organisms and all aspects of wastewater treatment systems.

2.6. Environmental Requirements for Nitrification:

To remove ammonia from wastewater, nitrifying bacteria found in the freshwater, groundwater, and sea are used. Nitrifying bacteria are found wherever their necessary nutrients, ammonia and oxygen exist. Due to their high requirement of nutrient, it's difficult to sustain them. For

their optimal performance of nitrifying bacteria should be provided, with the correct pH, dissolved oxygen concentration, temperature, hydraulic retention time, the presence of others nutrients for their growth (Rogers, 1983). Additionally, substances inhibitory to nitrifying activity must be removed, the pH should be between 7-8, the temperature optimally between 20-35°C, and dissolved oxygen above 2 mg/l. (Gupta & Sharma, 1996). Also, the COD needs to be removed to avoid competition between the autotrophic nitrifiers and heterotrophic bacteria (Grady, Daigger, & Lim, 1999).

Table 3: Summary of factors environmentalist influencing nitrifications (S. M. Zhang et al., 2015)

Factor	Optimum condition
Mixed liquor suspended solids	Provides an estimation of biomass concentrations between 500 and 5000 mg/L.
Alkalinity and pH	Optimal range for metabolism is between 7.2 and 8.9 Influent pH is important.
Dissolved oxygen	Provides information concerning the biological activity of the bacteria. The DO concentration is in the range 1.5- 2.0 mg/L in wastewater treatment.
Temperature	The optimal temperature is between 28-32 ^{o}C. At temperature below 5 ^{o}C and above 45 ^{o}C, nitrification does not occur.

2.6.1. Effect of free ammonia and ammonium concentration and pH:

The optimal pH for the nitrification process is between 7.2 and 8.9 (Quinlan, 1984). Therefore, below pH 7.2, the overall process can fail. The pH is important for the concentration of ammonia and nitrate because *Nitrosomonas* and some chemolithotrophic AOB used free ammonia rather than ammonium for their energy. At higher pH, the concentration of ammonia is high whereas the concentration of nitrous acid is low. Anthonisen et al (1976) stated that the non-ionized form of ammonia and nitrous acid may inhibit the nitrifying organisms. According to this statement, he found how to stabilize nitrification in a combination of pH. An alkaline pH equal to 7.5 is favorable especially for AOB. Most of nitrifying bacteria cannot grow at pH below 4 and above 9.5. Several operational problems may occur during biological treatment below the pH 6.8 and above the pH 7.5 (Gerardi, 2003): inhibition of nitrification, decreased of enzymatic activity, increased of production of hydrogen sulfide H_2S, undesired growth of some Nocardioforms and fungi, increased of ammonia production and interruption of floc formation (Gerardi, 2003). Chemolithotrophic AOB may be sensitive to ammonia depending on their physiological nature. Suwa and al (1994) discovered that AOB are typically sensitive to an upper concentration of ammonia in the sewage whereas they tolerate ammonia at high

concentrations in a reactor. The accumulation of nitrite due to the sensitivity of nitrite oxidation to free ammonia, which inhibits ammonia oxidation and nitrite oxidation (Anthonisen et al., 1976). In principle, the nitrite accumulation occurs when AOB grow faster than NOB. Unbalanced activity between AOB and NOB is observed at high pH, (Isaka, Yoshie, Sumino, Inamori, & Tsuneda, 2007) at low concentration of dissolved oxygen (Tokutomi, 2004) and at high temperatures (Van Dongen, Jetten, & Van Loosdrecht, 2001; Volcke, Van Loosdrecht, & Vanrolleghem, 2006) due to the presence of free ammonia. According to many researchers, most of nitrifying bacteria have an optimal pH between 7.5 and 8 (Painter and Loveless, 1983 citing Loveless and Painter, 1968; Prosser, 1989). Prosser (1989), in his study found out that *Nitrosomonas europea* a specie of AOB growth in the pH between 5.8 to 8.5. He stated also that a diversity of strains could exist within the species, for that the optimum pH for nitrifier in wastewater depends on the type of species. Since the temperature affects both the growth and the activity of bacteria, the optimal pH for growth and for the activity of bacteria will not be the same too (Barnes and Bliss, 1983 citing Loveless and Painter, 1968 and Painter, 1970). The optimal pH for nitrification activity range to 7.5 to 8.5 (Metcalf and Eddy, 1991a; Painter and Loveless, 1983 citing Downing *et al.*, 1964; Sedlak, 1991), and below pH 6 or above pH 10, no nitrification has been observed (Groeneweg et al, 1994; Painter and Loveless, 1983; Painter and Loveless, 1983 citing Downing et al, 1964). Specifically, the optimum pH for nitrifying bacteria is not the same. For example, for the case of *Nitrosomonas* (an AOB specie), the optimum pH for their activity is found equal to 6.7 to 9.2 whereas for Nitrobacter (a NOB specie), their optimal pH ranges for 8 to 9.5 (Churchwell *et al.*, 1980 citing several references; Wild *et al.*, 1971 citing several references). However, an acclimation of pH outside of the optimal range has been revealed possible for nitrifying bacteria. For that reason, this acclimation should be expected if some change has been made incase of experiment where the nitrifying bacteria will adapt to the environmental change condition (Barnes and Bliss, 1983). Some researchers have been achieved to acclimate an autotrophic nitrifier at pH above 11.2 and below 3.2 (Groeneweg *et al.*, 1994 citing Focht and Verstraete, 1977, Pennington and Ellis, 1993). However, the pH value in wastewater will depend on the production of nitrate (nitrification) or on its reduction to nitrogen gas (denitrification). And following that process, the bacteria involved during these reactions will acclimate or die according to their resistance. But unfortunately, that problem has not arisen during this experiment.

2.6.2. Effect of dissolved oxygen concentration:

Nitrifying bacteria are more sensitive to DO than others heterotrophs found in wastewater (Cheremisinoff, 1996). They required a high half-saturation of oxygen ($K_{S,O2}$) in the range between 0.1-0.2mg/l (Benefield & Randall, 1981) therefore, nitrifiers are susceptible at low concentrations of

DO (Prosser, 1989) and nitrification is inhibited. Some authors disagree about the minimum level of DO concentration, but it has been agreed that optimum nitrification can occur between 0.2-0.5 mg/L OF DO (Gray, 1989). Many literatures suggest that nitrite oxidizers are more sensitive than ammonia oxidizers at low concentration of DO. Dangcong et al, (2000) found that low DO concentrations inhibit nitrite oxidizers more than ammonia oxidizers. He reported that ammonia oxidizers have the ability to tolerate the fluctuation of DO concentration, but nitrite oxidizers do not. He used a sequencing batch reactor (SRB) in his study with two different methods of oxygen supply. The one was controlled and the DO was maintained in the range of 2-3 mg/l. In the uncontrolled oxygen supply, the concentration of DO had been changed. When the oxygen is controlled and the DO fixed at 2-3 mg/l, ammonia and nitrite occurred at the same time. But when oxygen supply is not controlled, nitrite oxidation is inhibited whereas ammonia oxidation proceeds. This is due to the fact that nitrite oxidizers have lower affinity for oxygen than ammonia oxidizers and have the difficulty to adapting to low oxygen concentration.

Since nitrification is an oxidation process, it requires oxygen, where dissolved oxygen is the major parameter to maintain stable nitrification as well as pH and over parameter. At low DO concentration, the nitrifying rate is low (Quinlan, 1984). Low DO and K_{S,O_2} during continuous nitrification will wash out the nitrifiers from the process, which will be replaced by non-nitrifying organisms. Thus, low K_{S,O_2} could result a failure of the nitrification process. The physiological adaption of nitrifying population exposed to a long lower concentration of DO should not be ignored. Different affinities have been observed between AOB and NOB in nitrification process. This may explain why NOB activity is lower at low DO concentration whereas AOB activity was not easily suppressed. Thus, DO is another key parameter, which influences nitrite accumulation (Tokutomi, 2004).

2.6.3. Effect of temperature:

Temperature is a factor for the growth of nitrifying bacteria (Sedlak, 1991). It has influence on the metabolic rate of nitrifying bacteria involving the population growth. Arrhenius formulated the basic equation between temperature and reaction rate in 1889, following this equation:

$$k_T = k_{20}\,\theta^{T-20}$$

Where k_T = reaction rate coefficient at temperature T, d^{-1}

k_{20} = reaction rate coefficient at $20^{o}C$, d^{-1}

θ = temperature-activity coefficient

T = temperature, ^{o}C

An optimal temperature has been observed for any kind of nitrifying bacteria. But, apparently, the optimal range of temperatures for growth will not be the same (Charley, Hooper, & McLee, 1980). The table below shows the relationship between nitrification and temperature.

Table 4: Relationship between temperature and nitrification (Gerardi, 2003)

Temperature (^{o}C)	**Effect on nitrification**
<5	Ends of nitrification
10	20% of nitrification rate
16	50% of nitrification rate
28-32	Optimal range of temperature
>45	Ends of nitrification

The nitrification process occurs in a wide range of temperature. Poduska and Andrews (1975) found in their research that the nitrification rate decreases with decreasing temperature. The effects of temperature on nitrification process are related to the slow growth of nitrifiers at low temperature (Ydstebø, Bilstad, & Barnard, 2000). Several studies assessed the influence of temperatures to the nitrification process (Poduska & Andrews, 1975; Ydstebø et al., 2000)

Like pH and DO, the temperature controls the growth rate of nitrifying organisms (Prosser, 1989). At increased temperature, the sludge retention time (SRT) of nitrification decreased progressively. At lower temperatures such as 7°C, nitrifying activity is still present. At higher temperatures, the maximum growth rate of AOB exceeds that of NOB (Van Dongen et al., 2001). Thus, at higher temperature between 30-35°C and with short SRT (1day), NOB could be removed from the microbial population, while AOB are retained. In the study conducted by Wild et al, (1971), it was shown that nitrification can occur at all temperatures between 5-30°C. To complete nitrification during cold season, it may be required to increase the SRT (Wild Jr et al., 1971). In another study, complete nitrification was observed in a pilot-scale activated sludge system with a SRT 15 days and temperatures 10°-15° and 20°C. While a partial nitrification was observed with a SRT 5 days at temperature 10°C (McClintock, Randall, & Pattarkine, 1993). To maintain complete nitrification at 10°C, a SRT between 5 and 8 days is necessary. A study of nitrification at low temperatures was demonstrated by Ydstebo et al, (2000) in a Norwegian BNR. In their study, it was demonstrated that an effective nitrification can be accomplished at low temperature such as 6°C with high SRT to

maintain nitrifiers growth. The literature suggests that increasing the SRT can minimize the impacts low temperature during nitrification process.

2.6.4. Substances inhibitory to nitrification:

Many compounds found in wastewater may have adverse effect on the nitrification process either directly or indirectly (Eilersen, Henze, & Kløft, 1994; Hanaki, Wantawin, & Ohgaki, 1990; Ichihashi, Satoh, & Mino, 2006). Several compounds inhibit nitrifiers, and it is required to lower the BOD stabilize nitrification. Several heavy metals like Cd, Cu, Zn, Pb and Cr are less toxic in the nitrification process (Madoni, Davoli, & Guglielmi, 1999). Park et al (2003) discovered that, the presence of zeolite in the wastewater might help recover nitrifying activity caused by Zn, because zeolite has the ability to adsorb zinc. Generally, wastewater containing higher or equal to 1% of salt will alter or inhibit the nitrifying community (Furukawa, Ike, & Fujita, 1993). Aside from heavy metals, others compound such as phenol, cyanide and thiocyanate are very toxic for nitrification. However, despite of the toxicity of phenol in the nitrification, it can serve as an electron donor during the denitrification (Yamagishi, Leite, Ueda, Yamaguchi, & Suwa, 2001). Cyanide and thiocyanate, they can be degrading by certain bacteria to produce carbonate, sulfate and ammonium. To conclude, all chemicals that may interfere the nitrification process need to be removed from wastewater.

2.7. Methodology:

Due to the toxicity of nitrate and nitrite and its impact to the human health and environmental, the determination of its concentration is essential for environmental protection and quality control. The detection and analysis of nitrate and nitrite is considered to be important. They can be determined by diverse methods explained below including also ion chromatography and ion electrophoresis.

2.7.1. Determination of nitrate:

Diverse standard methods are available to determine nitrate in wastewater samples. The analysis should be prompt and occurred within 24 hours of sampling and the samples should be stored at 4°C. Samples are usually conserved with acid and stored at 4°C if longer periods of storage are needed. Acid conservation will convert nitrates in the sample to nitrite. Consequently, samples that have been conserved with acid will not allow nitrate and nitrite to be determined separately. Only the total nitrogen can be determined from an acid conserved sample. The analytical standard methods used to measure nitrate in wastewater are: zinc reduction method, automated zinc reduction method, cadmium reduction flow injection, automated hydrazine reduction method, ultraviolet spectrophotometric screening method, nitrate electrode method, ultraviolet nitrate screening method (APHA, 1981).

Ion exchange chromatography (or ion chromatography, IC) is a subcategory of liquid chromatography. This method consist the separation of ions and polar molecules based on their charge. Ion chromatography utilizes the same material similar to liquid chromatography. It consists of a liquid mobile phase, a detector, a separation column, that measure the species eluted from the column. Ion-exchange chromatography can be used to determine ionic solutes, such as inorganic anions and cations, metals, and low molecular weight of acids and bases. It can also be applied for all kinds of charged molecule such as large proteins, small nucleotides and amino acids. The IC technique is frequently used to identify and quantify ions in various matrices. It can provide a suitable theoretic separation of anions. However, a high concentration of one anion present in the sample may interfere the detection of others ions. This method can be used in the laboratory but not suitable for online analysis.

The ultraviolet spectrophotometric screening method is used for screening samples, which are uncontaminated or have low organic matters contents. The determination of nitrate is measured by UV absorption at 220nm. However, a second measurement should be made at 275nm to correct the nitrate value, as nitrate does not absorb at 275nm. Hence, this method is not suitable if there is a significant correction required for nitrate. Acidification with 1N HCl is recommended to avoid interference from hydroxide or carbonate. The apparatus used in these methods is spectrophotometer (APHA, 1981).

The ultraviolet nitrate screening method is however similar than the previous method. Except, measuring the absorbance of nitrate at one wavelength is not possible because others organics matters absorbs also UV light. The range of organic matter from different water sources is not the same, so the UV nitrate screening procedure may not present reliable results (APHA, 1981).

For the case of nitrate ion electrodes, they are subject to interfere from nitrite, bicarbonate and chloride and some others anions. Ion electrodes may also provide erratic results. To maintain a constant pH and ionic strength, buffer solutions must therefore be used. The apparatus used in these methods are pH meter and double-junction reference electrode and nitrate ion electrode (APHA, 1981).

The zinc reduction method and automated zinc reduction method, prior analysis will convert nitrate to nitrite in the presence of zinc. Then, the nitrite produced will be determined by diazotizing with sulfanilamide and N-1-naphtyl-ethylenediamine-dihydrochloride to create a colored azo dye, in which the color will be measured via spectrophotometer. For this method nitrate needs to be measured shortly after sampling if we want to determine nitrate separately from nitrite. Theses methods could be automated and sometimes they can introduce toxic by products into the samples. The apparatus used in these methods are reduction column, colorimetric equipment and automated analytical equipment (APHA, 1981).

Concerning the automated hydrazine reduction method, the method is similar than the previous

but the $NO3^-$ is reduced to $NO2^-$ in presence of hydrazine sulfate (APHA, 1981). And the last one, in the cadmium reduction flow injection method; nitrate is converted to nitrite by placing the sample through a copperized cadmium column. And then the nitrite produced plus the nitrite present in the sample will be determined by diazotizing the nitrite with sulfanilamide and N-1-naphtyl-ethylenediamine-dihydrochloride. The solution will give a magenta color and the absorbance of the color will be measured at 540nm. The sum of nitrite produced and nitrite present naturally in the samples are known as total oxidized nitrogen (APHA, 1981). Additionally, the reagents used for all of these methods are variable depend on which method used.

Table 5: Summary of methods determining nitrate adapted from (APHA, 1981)

Methods	Apparatus	Reagents
UV spectrophotometric screening	Spectrophotometer	Nitrate-free water, stock nitrate solution, hydrochloric acid solution
UV nitrate screening	Spectrophotometer	Nitrate stock solution, nitrate-free water
Cadmium reduction method	Reduction column, filter photometer, colorimetric equipment	Copper-cadmium granules, color reagent, copper sulfate reaction, ammonia chloride EDTA
Automated cadmium reduction	Automated analytical equipment	Copper sulfate solution, copper-cadmium granules, hydrochloric acid, ammonium hydroxide
Nitrate ion electrodes	pH meter, double junction electrode and nitrate ion electrode	Nitrate-free water, stock nitrate solution, standard nitrite solution, sodium hydroxide
Cadmium reduction flow injection method	Flow injection equipment, absorbance detector	Ammonium chloride buffer, sulfanilamide color reagent
Automated hydrazine reduction method	Automated analytical equipment	Color developing reagent, copper sulfate stock solution, sodium hydroxide, hydrazine sulfate, standard nitrate solution

2.7.2. Determination of nitrite:

Nitrite can be determined by several methods. The colorimetric method requires the addition of

sulfanilamide and *N*- (1-naphthyl)-ethylenediamine dihydrochloride to produced a reddish purple color that can be measured photometrically. However, the ion chromatography method is subject to interference. The appropriate range of the spectrophotometric method is 10-to 1000 µg NO_2^-- N/L. It can be made in the range 5 to 50 µg N/L if a 5-cm light path and a green color filter is used and absorbance is measured at 543 nm. Colored ions that modify the color system should be removed. Suspended solids should be removed by filtration. Chemical incompatibility may allow the coexistence of NO_2^-, free chlorine, and nitrogen trichloride (NCI_3). Also, NCI_3 gives a false reddish purple color when reagent is added. Moreover, it is recommended to never use acid conservation for samples to be analyzed for NO_2^-. The analyses should be shortly after sampling and stored at 4^oC.

a. Spectrophotometric determination of nitrite:

Many methods have been described for the spectrophotometric to determine nitrite (Tomiyasu, Konagayoshi, Anazawa, & Sakamoto, 2001). For this, two methods are available for the determination of nitrite: nitrosation and Griess method (Qader, 2013). The most method used for the determination of nitrite is the Griess method. In this method, nitrite is treated with sulfanilamide, in an acidic media and will form a temporary diazonium salt. Then, this transient will react with a coupling reagent *N*- (1-naphthyl)-ethylenediamine dihydrochloride (NED) to form a stable azo-dye compound. The overall reaction is summarized in the figure below (Sun, Zhang, Broderick, & Fein, 2003):

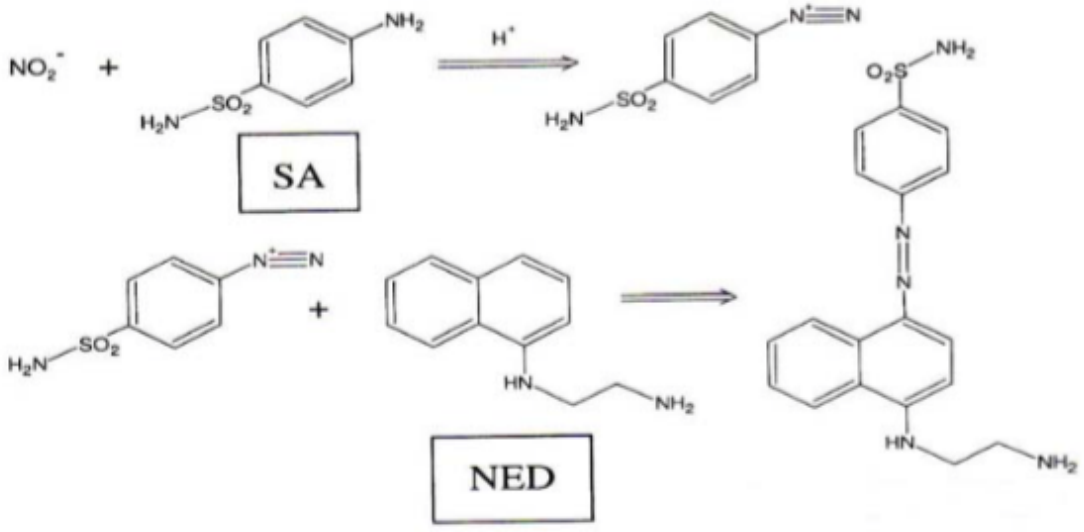

Figure 4: Overall reaction of sulfanilamide coupled with NED copied from (Sun et al., 2003)

b. Flow-injection method:

Flow-injection method is among the method used to determine nitrite in wastewater. This method is based on diazotization and coupling reaction (K. Higuchi & Motomizu, 1999). Staden and Makhafola (1996) established a simple spectrophotometric method to determine nitrite using flow-injection

analysis. Nitrite is diazotized with N-1-NED to produce a color azo dye that is quantified at 540 nm. In the spectrophotometric method, the procedure is similar like using the Griess method but 4-aminosalycylicis is used in acidic media instead of sulfanilamide, and then the diazonium produced will be coupled with N-1- NED yielding a reddish purple color. The absorbance of the color is measured at 515 nm. This proposed method enables the determination of 0.05 μg/ml of nitrite in wastewater with a sampling rate 180S/h and standard deviation 1%. Moreover, Mahmoud and Ali suggested the flow-injection technique to determine nitrite in wastewater. This method consist on the merging the nitrite ions in distilled water acidified with a carrier current flows N, N-dimethyl aniline and a stable complex was developed with an absorption maximum at 431nm. The injected sample volume was 65 ul of solution of nitrite ions, the rate of measurement of samples was 90 / h, and the detection limit was found to be 0.05 ug / ml (K. Higuchi & Motomizu, 1999).

c. Chemiluminescence method:

Chemiluminescence system is used for the determination of nitrite. This device is composed with 3 systems: liquid-gas separator, ozone generator and chemiluminescence reaction cell. Generally, this method is based on liquid-phase or gas-phase reaction between nitric oxide and ozone, resulting from the reduction of nitrite combined with iodine in sulfuric acid solution. The effectiveness of this system was evaluated by examining the analytical performance of the system for the determination of nitrite in batch and flow injection procedures. Under optimal conditions, the response of the system was linear against the concentration of nitrite over the range 0.001-10.0 μg/ml in the batch procedure and 0.01 – 5.0 μg/ml in the flow injection procedure, with the detection limits of 0.001 μg/ml and 0.01 μg/ml, respectively (Amini, Pourhossein, & Talebi, 2005). Consequently, this method allows the determination of nitrite in the presence of high concentration of nitrogen species (Amini et al., 2005). Under optimal conditions, the response of the system was linear against the concentration of nitrite over the range 0.001-10.0 μg/ml in the batch procedure and 0.01 – 5.0 μg/ml in the flow injection procedure, with the detection limits of 0.001 μg/ml and 0.01 μg/ml, respectively (Amini et al., 2005).

2.7.3. Quantification of nitrifying bacteria:

The diversity of nitrifying organism plays an important role in wastewater. To monitor and identify the content of nitrifying organism, suitable techniques have been created (Rittmann & McCarty, 2012). The analysis of the diversity of bacteria has been based on culture dependent techniques, which uses a diversity of culture media to permit the recovery of organisms. This technique has been used before the establishment of DNA and RNA based molecular techniques. Nonetheless, the culture-dependent technique is not suitable to quantify the diversity of bacteria. For instance, it has been demonstrated

that 0.01-1% of the total cell could be identified by plate counts (Amann, Ludwig, & Schleifer, 1995) and it is recognized as the "great plate count anomaly". Thus, most probable number (MPN) is among the method used for the enumeration of nitrifying bacteria (AOB and NOB) in different environment (Juretschko et al., 1998). However, for the case of Anaerobic Ammonia Oxidizing Bacteria (AnAOB), pure cultures are not available and the enrichment culture of AnAOB will take 100 to 200 days (Strous & Jetten, 2004). The quantification of bacterial numbers by the culture-dependent and MPN are difficult and unreliable because the maximum growth yields of nitrifying bacteria are low (Junier et al., 2010). Likewise, the culture independent such as the molecular techniques are more accurate and sensitive compared to the culture based method. Because of these problems of culture-based methods, the microbiologist has moved towards the molecular tools to determine and quantify the abundance and the diversity of nitrifying bacteria. An overview of these molecular methods will be explained below and summarize in the figure below. These molecular biological tools include Fluorescent in situ Hybridization (FISH), real time quantitative PCR (qPCR), microarrays, Polymerase Chaine Reaction (PCR), Denaturing gradient gel electrophoresis (DGGE) and T-RFLP (Jin, Zhang, & Yan, 2010).

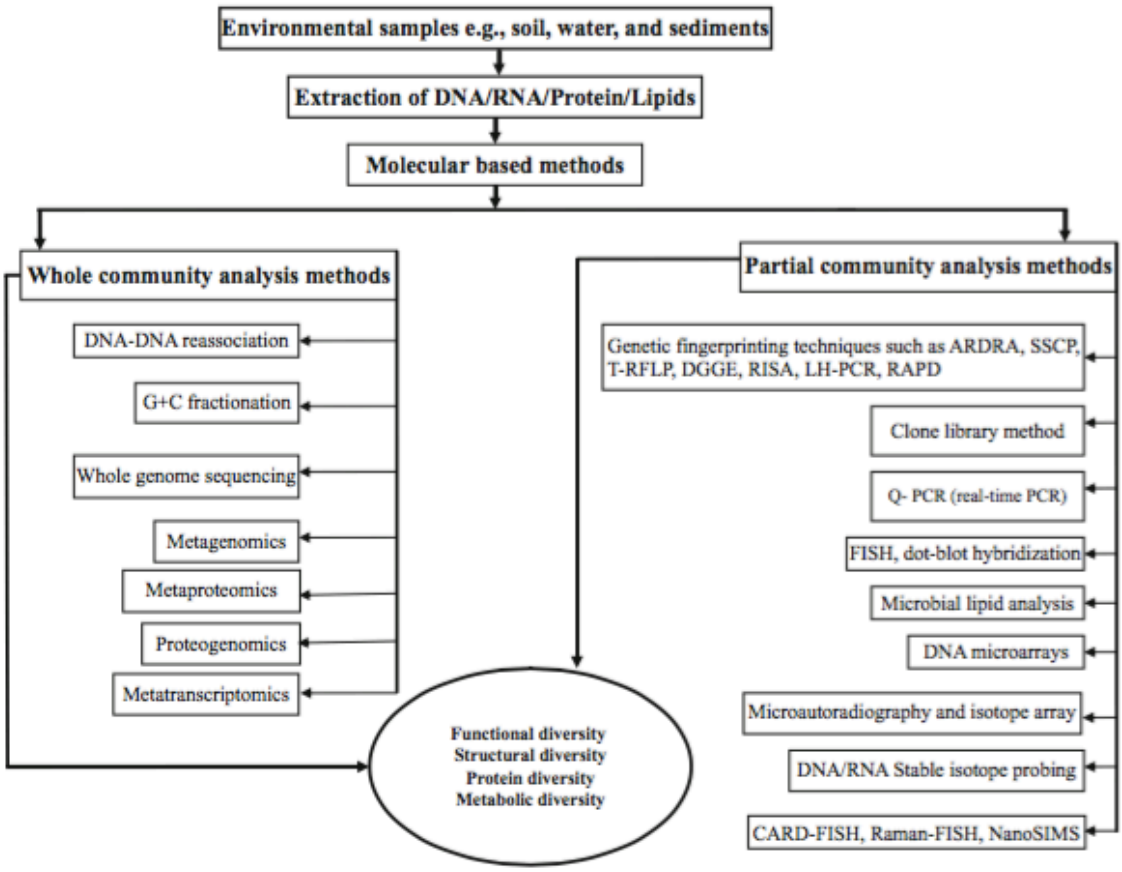

Figure 5: Culture-independent molecular toolbox to describe the diversity of bacteria in the environment copied from (Rastogi & Sani, 2011)

2.7.4. Polymerase Chain Reaction (PCR):

PCR is the initially step in culture-independent study for bacterial diversity present in a sample. It involves the amplification of DNA, throughout a number of denaturation, annealing and elongation in which DNA polymerase catalyzed the reaction (Saiki et al., 1988). This technique starts with DNA or RNA extraction to amplify the specific genes and will ends with phylogenetic analysis to identify the community diversity of nitrifying organism (Wahman, Kirisits, Katz, & Speitel, 2011). The product obtained in PCR is a mixture of DNA from different communities. This technique is useful for samples that contain a high concentration of inert bacteria with low activity or samples with high concentration of inactive particulates, which obstruct the application of FISH (Terada, Zhou, & Hosomi, 2011). The four main steps in PCR are:

- Denaturation, in which the extracted DNA is heat-denatured at 95°C or higher for 15 seconds to 2 minutes (Erlich et al., 1991) with synthetic oligonucleotide primers, which will flank the target DNA (Rodriguez Caballero, 2011).
- Annealing, in which the primers anneal to the DNA when the mixture product cooled down to approximately 40-60°C (Rodriguez Caballero, 2011).
- Elongation, in which DNA polymerase extends the primers with the DNA as template. Then, the mixture will be incubated (Rodriguez Caballero, 2011).
- At the end, the mixture is heated at approximately 74°C to isolate the strands and to let the primers to flank the new DNA strands. Approximately, 20-30 cycles could yield up to billion times DNA molecules (Madigan et al., 1997).

In PCR, the objective is not to replicate the whole strand of DNA but to replicate a target sequence. The components of the reaction are mixed and the reaction is placed in a thermal cycler, which is an automated instrument that takes the reaction through a series of different temperatures for varying amounts of time. The new synthesized DNA could be used as a new template. 1 million copies of a single piece of DNA can be made after 30 cycles in about 3 hours considering the time it takes to change the temperature of the reaction vial (Saiki et al., 1998).

2.7.5. Real time quantitative PCR (qPCR):

qPCR is now commonly used to determine the gene and transcript numbers of bacteria within samples (Smith & Osborn, 2009). This technique consists of the detection and quantification of fluorescence signal released in real time (R. Higuchi, Fockler, Dollinger, & Watson, 1993). Standard dilution series of known template concentration are required to get an absolute quantification of the target genes (Heid, Stevens, Livak, & Williams, 1996). These standards dilution could be a PCR amplified target DNA, a genomic DNA from pure culture or plasmids transporting a copy of the target gene. The

standard curve specifies a valuable method to determine the initial amount of the target template and to obtain the assay precision (Fraga, Meulia, & Fenster, 2008). Two different methods are known for qPCR: i) A probe based approach, most commonly TaqMan or molecular beacons and ii) approach based on double-stranded DNA (dsDNA) binding dyes, most commonly SYBR green. The last one is usually used to analyze environmental samples because it's low cost and low specificity compared to TaqMan and molecular beacons (Smith & Osborn, 2009). Nonetheless, its low specificity could be a weakness. Most of studies on the diversity and community of nitrifying bacteria via PCR and qPCR are based on targeting either the phylogenetic genes for example 16S rRNA or functional genes involved in the reduction of ammonia (Junier et al., 2010; H. B. Li et al., 2015). In this specific case, the focus will be on 16S RNA and functional genes in PCR and qPCR.

2.7.6. Denaturing gradient gel electrophoresis (DGGE):

In addition of molecular cloning and sequencing, DGGE is among the technique to analyze the diversity of microbial community. This approach is based on the separation of amplified DNA fragments with the same length and different sequences (Muyzer, De Waal, & Uitterlinden, 1993). The gel used in DGGE is an acrylamide gel with increasing concentration of a gradient DNA denaturant (a mixture of urea and formamide), or a linear temperature gradient (Muyzer et al., 1993). This technique allows the differentiation of DNA fragment of the same size based on their different denaturing properties. When the fragment DNA attain an area with ample denaturant in the gel, the gene will start to melt. Therefore, the melting properties of the genes will depend on the base sequence. Thus, the different bands of DGGE express different sequences according to the given gene, which is amplified via PCR (Madigan et al., 1997). After performing this method, the different bands can be removed from the gel and sequenced and phylogenetic analysis can be proceed. On the other hand, the DGGE method has some short comes. It is time consuming and expensive and difficult to define the mutation site in the DNA fragment (S. J. Ge et al., 2015).

2.7.7. Fluorescent in situ Hybridization (FISH):

FISH is among the biological molecular tools to identify and quantify the microbial cells of nitrifying organisms through cultivation-independent (Wagner, Rath, Amann, Koops, & Schleifer, 1995). This method binds a small fragment of DNA/RNA into a specific rRNA target in a morphologically cell by using oligonucleotide probe (Gieseke, Purkhold, Wagner, Amann, & Schramm, 2001). FISH detects the nucleic acid sequence through fluorescently labeled probe, which hybridizes particularly to its complementary sequence inside the intact cell. The techniques consist of sample fixation and hybridization to detect the target sequence and then visualization (Moter & Göbel, 2000). FISH has

been used to investigate microbial cells, the phylogenetic groups of the bacteria and the bacterial communities in wastewater as well as the distribution spatiotemporal. This method has been referred to be a standard method to detect anamox (Schmid et al., 2005). The main problem of this technique is the possibility of interference that could be reflected like auto-fluorescence and leads to false positive results (Moter & Göbel, 2000). The table 5 summarizes the list of the common probes used to detect nitrifying bacteria.

Table 6: Probes used for FISH to identify nitrifying bacteria

Group	Probes	Target	References
AOB	Nso1225	β-proteobacterial AOB	(Mobarry et al., 1996)
NOB	Nso190 Ntspa662 + competitor Nit3 + competitor	*Nitrospira sp*. *Nitrobacter sp*.	(Daims, Nielsen, Nielsen, Schleifer, & Wagner, 2001)
AnAOB	Amx820 Amx1240	*Kuenenia & Brocadia* *Brocadia anammoxidans*	(Schmid et al., 2005)

2.7.8. Theory and measurement of nitrification growth rate:

To study the bacterial growth of nitrifying bacteria, the SBR will be inoculated by a sterile culture media, which serve as nutrient for their growth. After feeding, the pH should be adjusted to be 7.5 and the samples are then kept at two different temperature: 10 and 25°C. Under these conditions, the bacteria will have all the requirements favorable to their development. When the nitrifying bacteria reach a certain range, they will split by binary fission, giving a daughter cell, in which one cell will split in two and two cells, will give four daughter cells and the process will follow the geometric approach. At this time, the nitrifying bacteria are in their growing phase. They will use the components of the culture medium and will reproduce quickly until the media will be exhausted or limited.

2.8. Objectives:

The aim of this master thesis is to determine the nitrification rate in wastewater by quantifying the nitrifying organisms present.

Objectives are:

-To procure a deep knowledge of nitrification in wastewater treatment, the nitrifying organisms, and the affecting factors inhibited the process.

-To finish a literature review study on aspects of nitrification rate in wastewater.

- Analyzing laboratory tests should accomplish this comparison.

III. Materials and Methods

The sludge used in the experiments during this master project was obtained from the wastewater treatment plant IVAR in Mekjarvik (Stavanger, Norway). Experiments were carried out in six sequencing batch reactors (SBRs), operated in parallel under aerobic condition.

3.1. Preparation and maintenance of the nitrifying mixed culture:

Each continuous aerobic SBR culture was made with a working volume of 1.5 L where an air pump was used to supply oxygen and a magnetic stirrer set to 50 rpm ensured mixing. The culture consisted of a mixture of 1.35 L of culture medium (Table 7), 150 mL of sludge from IVAR and 1 mL trace metal solution (Table 8). The experimental conditions were the same for all 6 reactors, expect for the incubation temperature. Three reactors (number 1, 2 and 3) were maintained at room temperature 25°C and three other reactors (number 4,5 and 6) were kept at 10°C. The reactors were feed twice a week: every Monday and Thursday. Prior to adding fresh culture medium, the culture was allowed to settle for at least 30 min then the liquid phase (approx. 1.35 L) was decanted and replaced with the fresh medium. The amount of air inflowing the batch reactor was automatically fixed using aquarium pump 200 for the oxygen supply and the pH was maintained at 7.5±0.3.

Ammonia, nitrate and nitrite were analyzed daily, whereas COD, VSS and TSS were analyzed after feeding the reactors. The pH of the samples was measured and recorded every morning in order to assess quickly their state.

Table 7: Nitrifying culture medium

Chemicals	Concentration of stock solution mg/L
K_2HPO_4	600
KH_2PO_4	335
$CaCl_2.2H_20$	67.5
$MgCl_2.6H_2O$	135
$MgSO_4.7H_2O$	267.5
$FeCl_2.4H_2O$	67.5
NH_4Cl	100

Table 8: Trace metal stock solution

Chemicals	Concentration of stock solution mg/L
$ZnCl_2$	25
$MnCl_2.4H_2O$	15
H_3BO_3	150
$CoCl_2.6H_2O$	100
$CuCl_2.2H_2O$	5
$NiSO_4.6H_2O$	10
$NaMoO_4.2H_2O$	15

3.2. Analysis of different nitrogen forms:

Nitrite, nitrate, total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed regularly following the standard methods (APHA, 1981) whereas ammonia and chemical oxygen demand (COD) were analyzed by using a test kit. All samples (except for TSS and VSS determination) were filtered through a 1.2 μm pore size filter prior to analysis. The pH of the samples was checked everyday using a pH meter (pH 730 inolab). The pH meter was calibrated before each analysis using the buffer solution pH 4 and 7. The suitable pH for the samples was 7.5 and was adjusted by adding necessary amounts of 2M of sodium hydroxide.

3.2.1. Ammonium analysis:

Ammonium concentration in the samples was measured daily by using 3 different Spectroquant cell test kits which were respectively, #114559 in the range of 4-80 mg/l NH_4-N, #114558 in the range of 0.20-8 mg/l NH_4-N and #114739 in the range of 0.010-2 mg/l NH_4-N (Merck, VWR), respectively. Analysis was carried out according to the protocol in each kit. Briefly, 5 ml sample was pipetted and reacted with Sodium nitroprusside (reagent NH_4-1K) to develop a blue color, which was then measured using a spectrophotometer (Pharo 300). The concentration of ammonium was determined at the wavelength 665 nm.

3.2.2. Nitrite analysis:

Nitrite concentration in the samples were determined according to the standard methods for the examination of water and wastewater (APHA, 1981). A colorimetric procedure was used to carry out this analysis. The buffer solution (Griess reagent) was prepared by mixing 12 mL of orthophosphate, 5 g of sulfanilamide and 0.5 g of NED in 250 mL of distilled water. It was stored in a dark bottle at 4°C and remained suitable for at least one month or until a brown color formed. The stock nitrite solution was prepared by dissolving 0.69 g of sodium nitrite in 100 ml of distilled water. A series of dilutions (0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 mM) were prepared from this standard by pipetting 0.5, 1.0, 2.0, 5.0,

8.0, and 10.0 ml and bringing up the final volume to 100 ml with distilled water respectively. To make the standard curve, 3 replicates of each dilution were prepared by mixing 12 ml of distilled water, 250 µL of the Griess solution and 50 µL of the stock nitrite solution according to their dilution. The standard dilutions were then vortex. After 15 min, the absorbance was measured using the spectrophotometer (Shimadzu UV/VIS) at 543 nm in small glass cuvettes. The absorbance of standards against the nitrite concentration was used to make the standard curve. The determination of nitrite concentration in the sludge followed the same procedure like as the standard solution, replacing the 50 µl nitrite stock with 50 µl of sludge sample.

3.2.3. Nitrate analysis:

The chemicals used during this analysis were sodium nitrate, 5% ammonium chloride, sulfanilamide, NED and Zinc metal 30 mesh. This quantification assay consists of the reduction of nitrate to nitrite using elemental zinc. Then the nitrite formed reacts with the Griess reagent and changing it to its diazonium salt forming a purple azo dye, which absorbs light at 543 nm. The stock nitrate solution was prepared by dissolving 0.85 g of sodium nitrate in 100ml of distilled water. Different dilutions (0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 mM) were prepared from the standard by pipetting 0.5, 1.0, 2.0, 5.0, 8.0, and 10.0 ml and bringing the volume up to 100ml with distilled water. After that, to make the standard solution, 3 replicates of each dilution was prepared by mixing, 6 ml of distilled water, 6 ml of 5% ammonium chloride, 100 µL of the stock nitrite solution according to their dilution and 0.3 g of zinc metal 30 mesh. The test tubes were shaken for 10 min to allow the solution to make contact with the zinc to reducing the nitrate to nitrite. After 10 min, the zinc was allowed to settle to the bottom for 5 min. Then, we decanted the solution to a new test tube while all of the zinc stayed in the original test tubes. After that, 250 µL of the Griess reagent was added to the solution and a purple color formed. The standard dilutions were then vortexed to allow the sample to mix well. After 15 min, the absorbance was measured using a spectrophotometer (Shimadzu UV/VIS) at 543 nm using small glass cuvettes. Plotting absorbance of standards against the nitrate concentration was used to make a standard curve. The determination of nitrate concentration in the sludge followed the same procedure like we did to make the standard solution and the final concentration of nitrate was obtained by subtracting its concentration with each sample of nitrite concentration.

3.3. Sludge properties and COD:

3.3.1. Chemical oxygen demand (COD):

COD is a method to determine the amount of organic matter in water, which is able to oxidize by a strong chemical oxidant. The test kit #14541 in the range of 25-1500mg/l was used according the manufacturer instructions. A sample (2 mL) was added to a test kit tube, which contained a mixture of mercuric sulphate, potassium dichromate, and sulphuric acid. The tubes were boiled at 150°C for two

hours. After boiling, the tubes were allowed to cool and COD values were obtained. Readings were taken for each tube using a spectrophotometer (Pharo 300, Merck).

3.3.2. Suspend solids:

Total suspended solids (TSS) are the amount of all solids in wastewater. TSS was determined by filtering 20 mL of the sample through a Whatman GF/C glass microfiber filter (1.5 μm pore size), which has been weighed to four decimal precisions prior to filtering (weight of filter). After drying the filter at 105 °C for 1 hours, the filters were put into a desiccator for 15min. After cooling in a desiccator, the filter was again weighed. TSS was finally determined by using the following formula:

$$\mathbf{TSS}=\frac{\text{Weight of filter+solids in the sample after drying at 103°C}-\text{Weight of filter}}{\text{Volume of wastewater sample}}$$

3.3.3. Volatile suspended solids:

Volatile suspended solids (VSS) are the amount of solid that volatilizes when combusted at 550 °C for 1 hours in a muffle furnace. Filters from the TSS analysis (containing dried solids from 20 ml of wastewater) were combusted (550 °C, 1 hour), cooled in a desiccator, and finally weighed to four decimal precisions. VSS was determined by using the following formula:

$$\text{VSS}=\frac{\text{Weight of filter + solids after drying at 103°C}-\text{Weight of filter + solids after combusting}}{\textbf{Volume of waste water sample}}$$

3.4. MPN method for enumeration of nitrifying bacteria:

The MPN is based on the presence or absence of bacteria forming units in tests the tubes. A media selective for nitrifying bacteria was used (Table 9) to monitor bacterial populations over a period of time, approximately 80 days of incubation. But during this project, the media tubes were incubated only for 60 days due to time constrains. Two MPN test were carried out, one at time 0 and one at 37 days (both incubated for 60 days). Stock solutions were prepared using the specified amounts listed in Table 9. The pH of the media was measured and adjusted to pH 7.2 using 2M of NaOH before distribution into the media tubes. An aliquot of 9 mL of media was transferred to each of 100 media tubes to be used for each sample. Five replicates for each dilution were made. All tubes were then capped and autoclaved at 121°C for 20 min. 1 mL aliquot of SBR sample was transferred to a sterile dilution tube containing 9 mL of the nitrifier media by using a sterile serological pipette. The solution was vortex mixed and marked 10^{-1}. An aliquot 1 mL of the diluted sample 10^{-1} was then added to the following test tubes. The solution was vortex and marked 10^{-2}. The same procedure was repeated up to a 10^{-10} dilution. Lasting for 60 days, labeled tubes media were capped and incubated at 10°C and 25°C. A change in color of the solution from blue to yellow indicated a decrease in the pH of the sample and nitrifier growth. Then, the tubes were tested for the presence of nitrite. To detect nitrite production in

the MPN samples, the Griess reagent solution was used. The method used to identify positive and negative results for nitrifying bacteria was to check any color change for all test tubes indicating the positive test of nitrite if not, nitrate spot test was performed. To perform that test 0.1 mL of the sample from each tube was aseptically transferred to a96 wells plate. A drop of the Griess reagent was added to the sample and the change in color pink to red indicated the presence of nitrite. For tubes with negative results, a drop of the nitrate spot test reagent was added to 0.1 mL sample in a spot plate. All samples producing color change after addition of the Griess reagent signifies positive results. Results were recorded and an estimation of the quantity of nitrifiers present was found by using the appropriate MPN table (Vikesland et al., 2007) shown in appendix A.

Table 9: Composition of stock solution for nitrifier media used for MPN method

Chemicals	Concentration of stock solution g/100 mL	ml of stock solution required per L of media
$CaCl_2 2H_2O$	1.34	1
$(NH_4)_2SO_4$	5	10
$MgSO_4 7H_2O$	4	1
KH_2PO_4	2.72	1
$FeSO_4 7H_2O$	0.246	2.5
EDTA disodium	2.72	1
Bromothymol Blue	0.04	5
Trace elements:		
$Na_2MoO_4\ 2H_2O$	0.01	1
$MnCl_2$	0.02	
$CoCl_2 6H_2O$	0.00002	
$ZnSO_4 7H_2O$	0.01	
$CuSO_4 5H_2O$	0.002	

3.5. Analysis of bacterial diversity of nitrifier:

3.5.1 DNA extraction:

All filtration equipment used prior to DNA extraction was washed and autoclaved to avoid contamination. DNA extraction was performed at time 0, 23 and 60 days by using a commercially available PowerSoil® DNA Isolation Kit (MoBio). 100 mL of the sample was filtrated for each temperature 10 and 25°C. The sample was vacuum filtrated via nitrocellulose membrane filters (0.2 µm GSWP, Millipore). Then, the filters were removed with tweezers and put into a sterile surface. The filter was then cut into small pieces by using sterile scissors and added to the PowerBead® tube

provided in the PowerSoil® kit. DNA extractions were performed according to the manufacturer's instructions following homogenization in a bead beater (MPI) at speed 6 for 1 minute.

3.5.2. PCR amplification:

The PCR amplification of 16S rRNA genes was achieved using the universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') and the reverse primer SD907-r 5'CCCCGTCAATTCCTTTGAGTT-3') with GC-clamp (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'), targeting the V3-V4 hyper variable region of the 16S rRNA gene (Brakstad & Bonaunet, 2006). The DNA sequences were amplified using an Applied Biosystems 2720 Thermal cycler. The PCR program was: denaturation for 10 min at 94°C, followed by 30 cycles for 30s at 94°C, annealing for 40s at 55°C, elongation for 1 min at 72°C and final elongation for 7 min at 72°C. The PCR products were allowed to cool down to 4°C. The composition of the reaction and PCR mix is listed in Table 10 and 11.

Table 10: Components of the reaction mix 8 (50 µl total volume)

Components	Concentration	Volume
Mol. grade dH_2O	-	39 µL
10x PCR buffer (5Prime)	10 mM tris HCl, 50mM HCl	5 µL
Template DNA		1 µL
PCR mix		**5 µL**

Table 11: Components of the PCR mix (for 10 samples)

Components	Concentration	Volume
Forward primer	100 µM	10 µL
Reverse primer	100 µM	10 µL
Taq polymerase	25 U/µL	5 µL
10 mM dNTPs	40 mM	5 µL
Mol. grade dH_2O		20 µL

3.5.3. Agarose gel electrophoresis:

Agarose gel electrophoresis is a technique to check the presence of DNA in the sample. To check the crude DNA obtained from the extractions, and to check the size and purity of PCR products, a 2% agarose gel was prepared by dissolving 1 g of electrophoresis grade agarose in 50 ml of 1x Tris-Acetate-EDTA (TAE) buffer (prepared from 50x stock, VWR). Then, the mixture was heated in a microwave oven until all agarose melted and dissolved. GelGreen dye (VWR) was added to the melted

agarose (5 µL of a 50000x stock) and this solution was then transferred into a gel rack containing combs to make the wells. The gel was then left to solidify within half hour. After that, the combs were removed, the gel was placed in an electrophoresis chamber and the chamber was filled with 1x TAE buffer. From the PCR reactions, 5 µl of the each sample was mixed with 2 µl gel loading dye and the mix was loaded on the gel. A DNA ladder was also loaded on each gel to be used as a marker for DNA fragment size (Direct Load, WideRange DNA ladder, Sigma). The gel was then run at 80 V for 90 min. At the end, the gel was visualized using a Gel Doc system (BioRad, GelDocXR), under UV light.

3.5.4. DGGE analysis:

Denaturing solutions (20 and 80%) were prepared with 6 % acrylamide content to make the DGGE gel (Table 12). The solutions were then stored refrigerated in a dark bottle until use. The gel casting frame was assembled and 30 ml of each (20 and 80%) denaturing solutions were transferred into a 50 ml centrifuge tube. To initiate polymerization, 30 µl of tetramethylethylenedimaine (TEMED) and 300 µl of a 10% ammonium persulfate polymersing (APS) was added to each of DGGE solutions, mixed well and poured immediately using a gradient former. The gel was poured between the glass plates of the device using a peristaltic pump running at 7 rpm. It was then left to solidify for at least 2 hours. Prior to loading the gel, 20 µl of sample (PCR product) was mixed together with 5 µl of DGGE gel loading dye. The gel was run at 90V for 18 hours at 60°C using the IngenyPhor U2 system filled with 17 L of 1x TAE buffer. For post-staining of the gel, GelRed (50000x stock VWR) was used (100 µl dye in 1 l TAE buffer). After 1 hour staining, the gel was visualized and the pictures were taken from the device BioRad GelDOC XR Imagery System.

Table 12: Composition of 20 and 80% denaturing solutions.

Compound	20% DGGE solution	80% DGGE solution
40% Acrylamide Bis	30 mL	30 mL
50x TAE buffer	4 mL	4 mL
Formamide	16 mL	64 mL
Urea	16.8 g	67.2 g
dH_2O	Up to 200 mL	Up to 200 mL

3.6. Determination of the day 60 (quasi steady-state) nitrification rate:

Nitrification is a biochemical reaction carried out by nitrifying bacteria, contributing to the disappearance of ammonia, and production of nitrite and nitrate in wastewater. The rate at which these processes occur in a steady-state nitrifying reactor can provide essential information for determining SRT of a nitrification unit in a wastewater treatment plant. To study the nitrification rate, of the mixed

culture developed after 60 days of incubation in the SBRs (originally inoculated with wastewater sludge) of this study, a time series measurement of ammonia, nitrate and nitrite concentration was carried out. This was done by taking samples from one reactor at each temperature every half an hour during an entire day (9 hour period) starting from just after feeding the SBRs. For determining ammonia, nitrite and nitrate, the same methods were used as described above.

3.7. Statistical analysis:

To determine the error that occurs during sample analysis, statistical analysis was performed via standard deviation of the mean. The mean $\bar{x}$ is the average value of a dataset from replicate measurements. For this purposes of this study, the arithmetic mean of values were used following this equation below. The standard deviation measures how much a parameter or community aspect deviates from the mean. For this study standard deviation was used to assess the variability of the data and to see the difference between measurements. If some of the deviation is large, our measurements are obviously not so precise. With this definition, the standard deviation can be described as the root mean square deviation of the measurement $x_1 \ldots x_N$ (Taylor, 1982).

$$\sigma = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(x_i - \bar{x})^2} \qquad \bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

IV. Results:

The objective of the SBR experiment was to quantify the fluctuation of ammonia, nitrite and nitrate concentration in wastewater, to determine the nitrification rate of the mixed culture developed after 60 days of incubation and the microbial diversity of nitrifying bacteria.

4.1. Ammonia, nitrite and nitrate measurement:

While ammonia was measured using a commercially available kit, the concentrations of nitrite and nitrate were determined using manually prepared standard curves. Figure 6 and 7 shows the calibration curves for nitrite and nitrate, respectively. Values of absorbance were the average of 3 replicate measurements. The equation obtained from the nitrite standard was y = 0.1708x - 0.0702 (R^2 = 0.99802), while the regression linear for nitrate standard curve was y = 0.0345x - 0.0015 (R^2 = 0.99586). From these equations, the concentration of nitrite and nitrate in the wastewater was calculated.

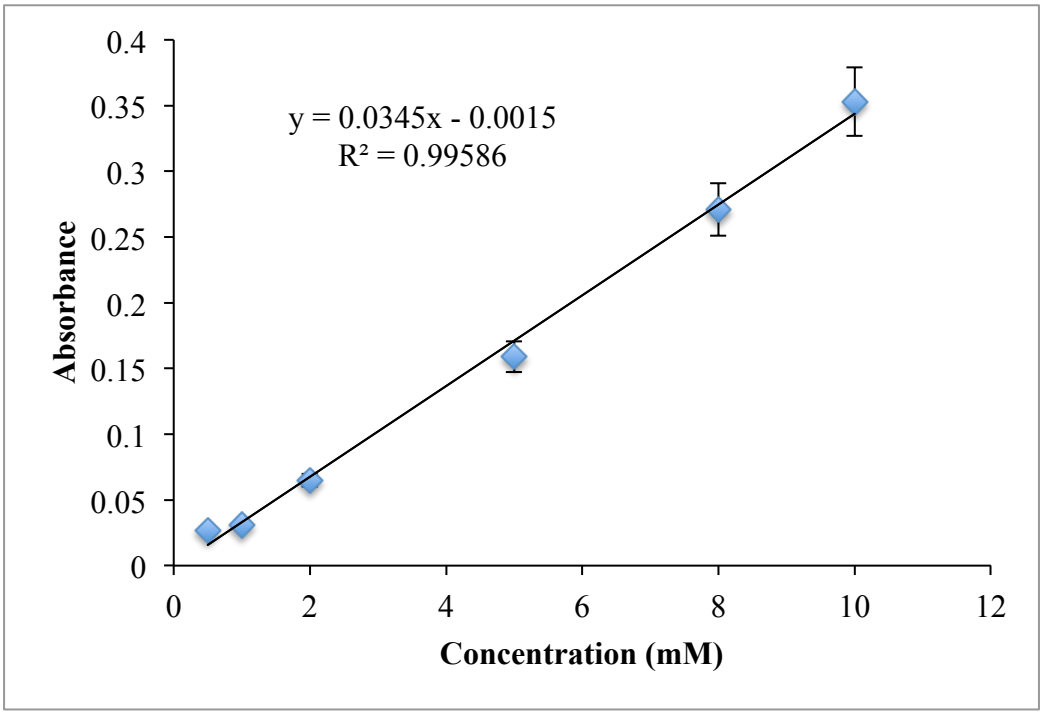

Figure 6: Calibration curve of standard nitrate. Mean of all calibration values used during the experiments shown as data points. Errors bars show the standard deviation

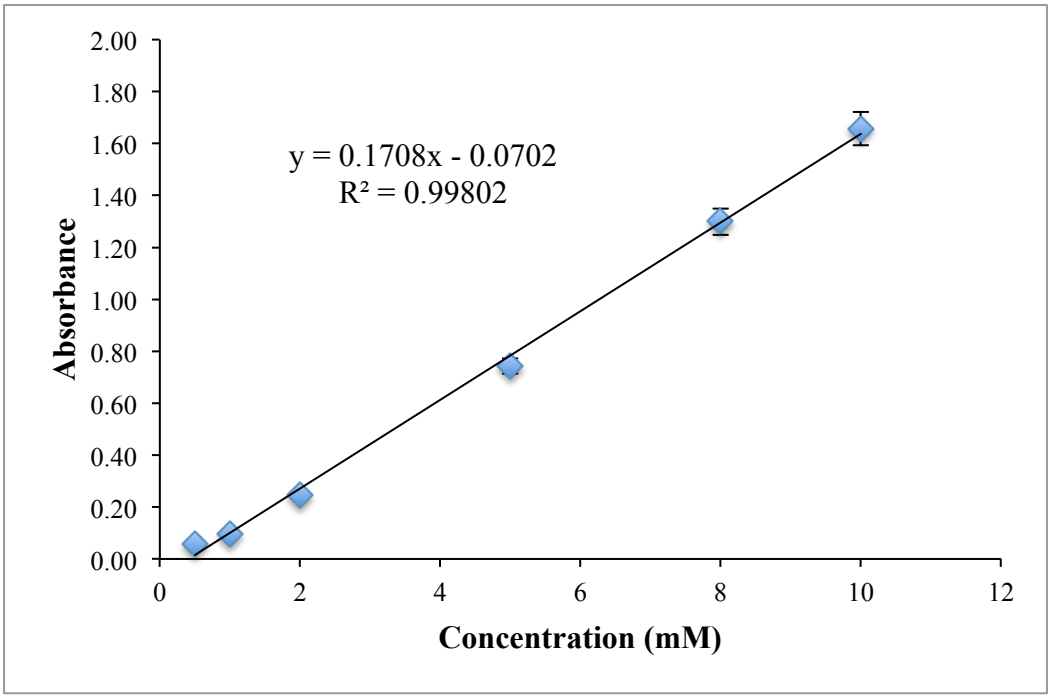

Figure 7: Calibration curve of standard nitrite. Mean of all calibration values used during the experiments shown as data points. Errors bars show the standard deviation.

The daily ammonia, nitrite and nitrate concentration measurement results during 60 days for 25°C incubation temperatures are summarized in Figure 8 and 9, while for the temperature 10°C, results are shown in Figure 10 and 11. The 60-day period was split into two parts (day 0-28 as Part 1 and day 29-60 as Part 2). During these experiments, the ammonia concentrations were expected to decrease due to nitrification whereas nitrite and nitrate would increase. During the beginning of the measurement, the change in ammonia, nitrite and nitrate concentrations showed favorable results. The ammonia concentrations at the beginning were higher and decline with increasing nitrite and nitrate concentrations. Also, the nitrite concentration never reached significantly high levels throughout the reaction, and it is assumed that the nitrite was quickly converted to nitrate within the SBR. It was very evident from Figure 8, 9, 10 and 11 that the concentration of ammonia, nitrite and nitrate became mostly constant within 60 days. These sampling results have shown that nitrification occurred at both 10 and 25°C. The ammonium, nitrite and nitrate levels were generally higher at the higher incubation temperature. The decline point of nitrite and nitrate concentration indicated the time when the reactors were fed, while the same time point is represented by a peak point in ammonia concentration. At higher temperature, a significant concentration of nitrate is observed while ammonia concentration decreased. Whereas at cold temperature 10°C, even though there is nitrification, nitrite and nitrate concentration were smaller. At 25°C, the ammonia concentration ranges from 21.6 mg/l to 0.27 mg/l with a SD 3.98 mg/l. Nitrite concentration ranged from 7.82 mg/l to 0.46 mg/l with SD 2.18 mg/l and nitrate concentration to 45.27 mg/l to 0.024 mg/l with SD 15.003 mg/l. However, at 10°C ammonia,

nitrite and nitrate concentrations were very low. Their concentrations ranged respectively from 9.3 mg/l to 0.2 mg/l for ammonia with SD 1.71 mg/l, 4.47 mg/l to 0.41 mg/l for nitrite with SD 0.96 mg/l and 10.51 mg/l to 0.005 mg/l for nitrate with SD 2.24 mg/l.

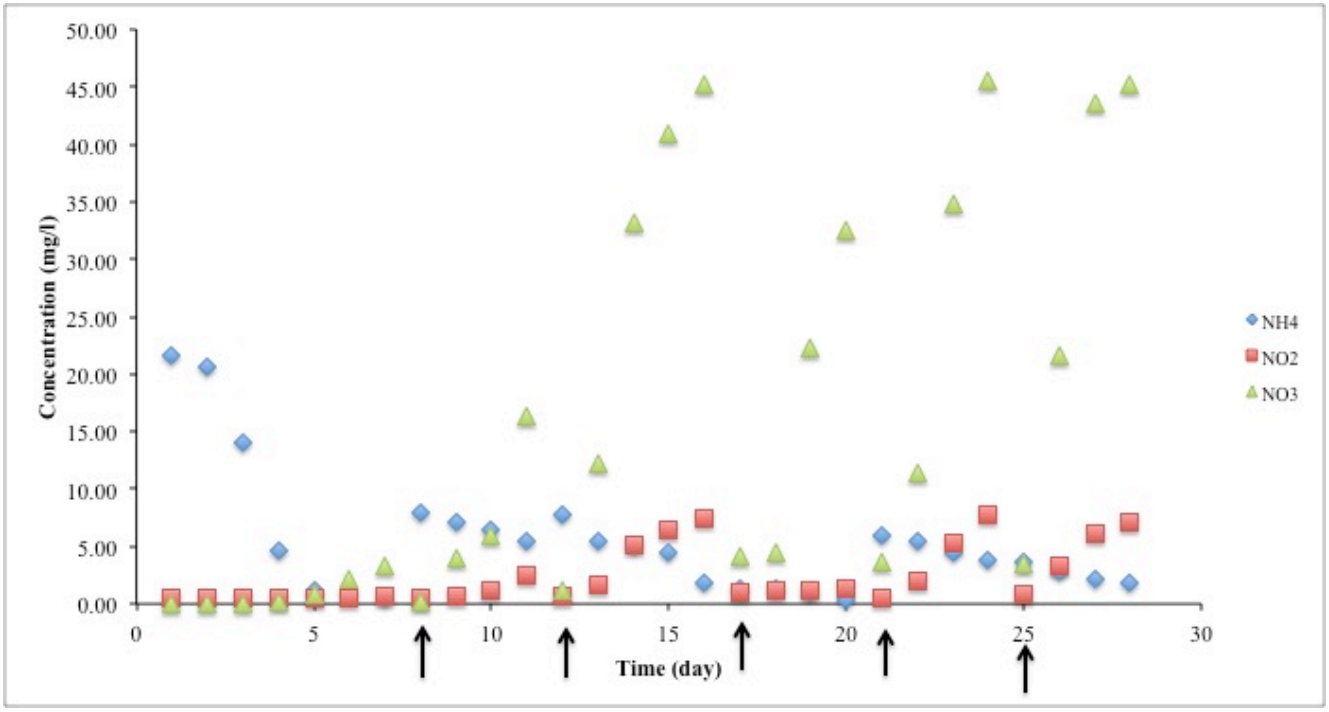

Figure 8: Variation of ammonia, nitrite and nitrate concentration at incubation temperature of 25°C from day 0 until day 28 (Part 1). Arrows show the time of feeding.

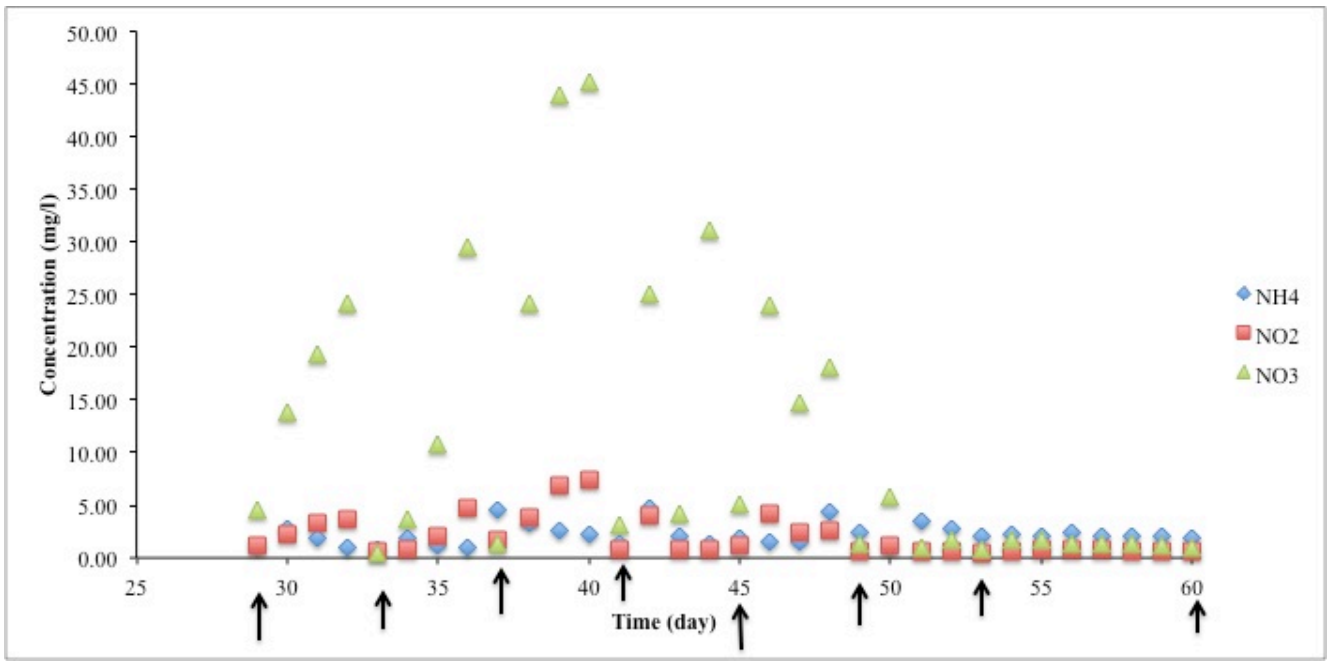

Figure 9: Variation of ammonia, nitrite and nitrate concentration at incubation temperature of 25°C from day 29 until day 60 (Part 2). Arrows show the time of feeding.

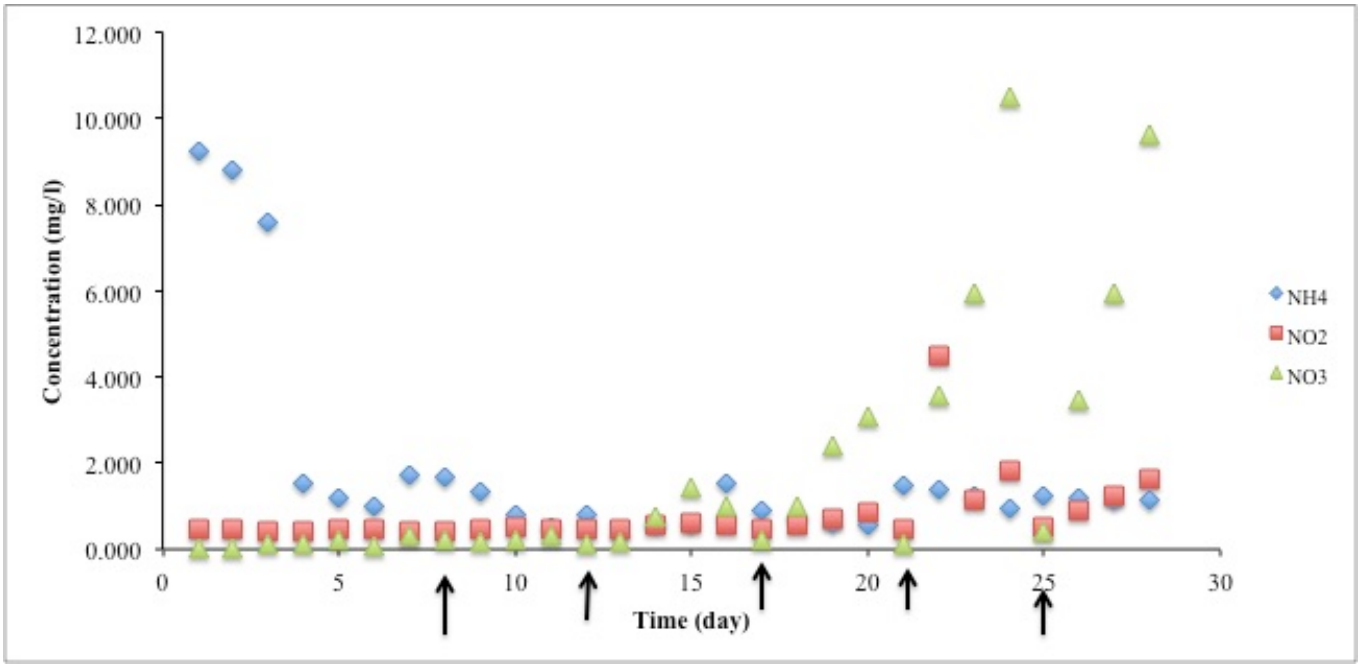

Figure 10: Variation of ammonia, nitrite and nitrate concentration at incubation temperature of 10°C from day 0 until day 28 (Part 1). Arrow Show the time of feeding.

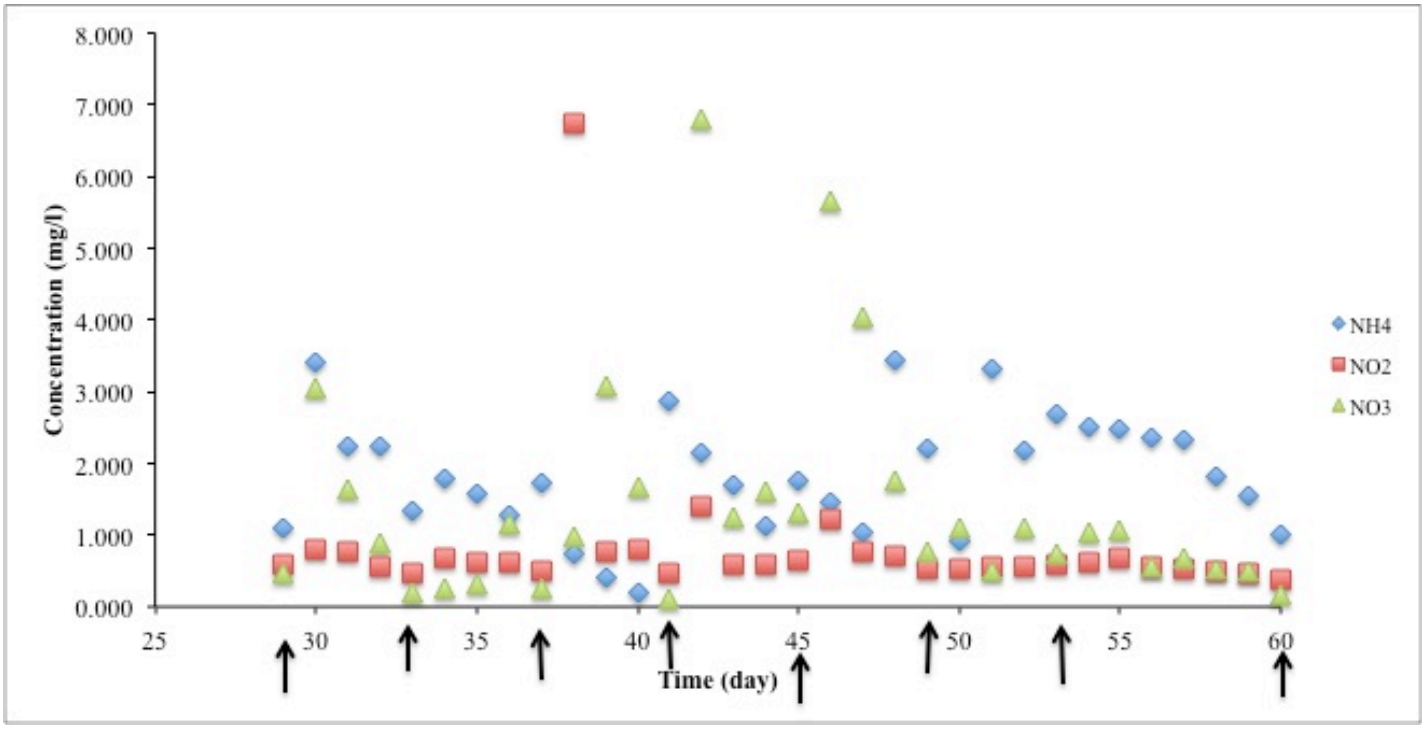

Figure 11: Variation of ammonia, nitrite and nitrate concentration at incubation temperature of 10°C from day 29 until day 60 (Part 2). Arrow show the time of feeding

4.2. Sludge properties:

Figure 12 shows the variation of TSS and VSS from this experiment. Based on our result, the average of TSS at 25°C was 0.622 ±0.4g/l and for the VSS we have obtained 0.373 ±0.34g/l. And at temperature 10°C, the TSS value range from 0.471 ±0.34 g/l whereas the VSS value range from 0.240 ±0.26 g/l. The maximum concentration of TSS and VSS at both temperatures were almost the same as it was the beginning of the analysis at time zero. Otherwise, all along these experiments, the figure below showed also that the TSS and VSS concentration are higher at 25°C than 10°C.

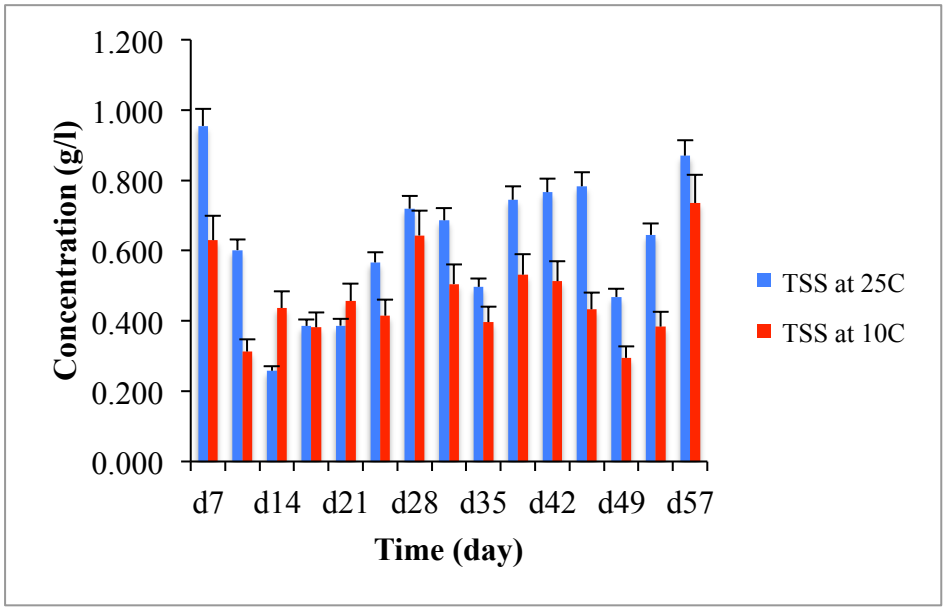

Figure 12: Total suspended solids (TSS) concentration at 25°C. Errors bars show the standard deviation.

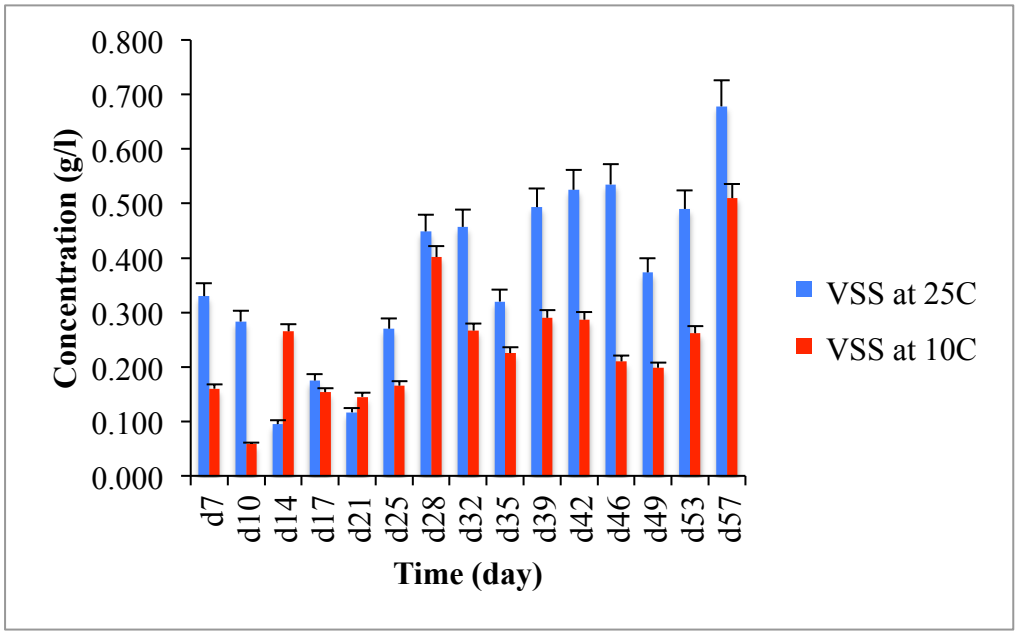

Figure 13: Volatile suspended solids (VSS) concentration at 10°C. Errors bars show the standard deviation

4.3. COD analysis:

In the present study, the average of COD concentration values range between 314 and 148 mg/L for the samples incubated at 25°C, and between 215 and 122 mg/L for the samples incubated at 10°C. COD values were clearly different at the two temperatures, the COD concentration was higher at 25°C than 10°C. Also, during the last two weeks of the experiments, the COD concentration became stable.

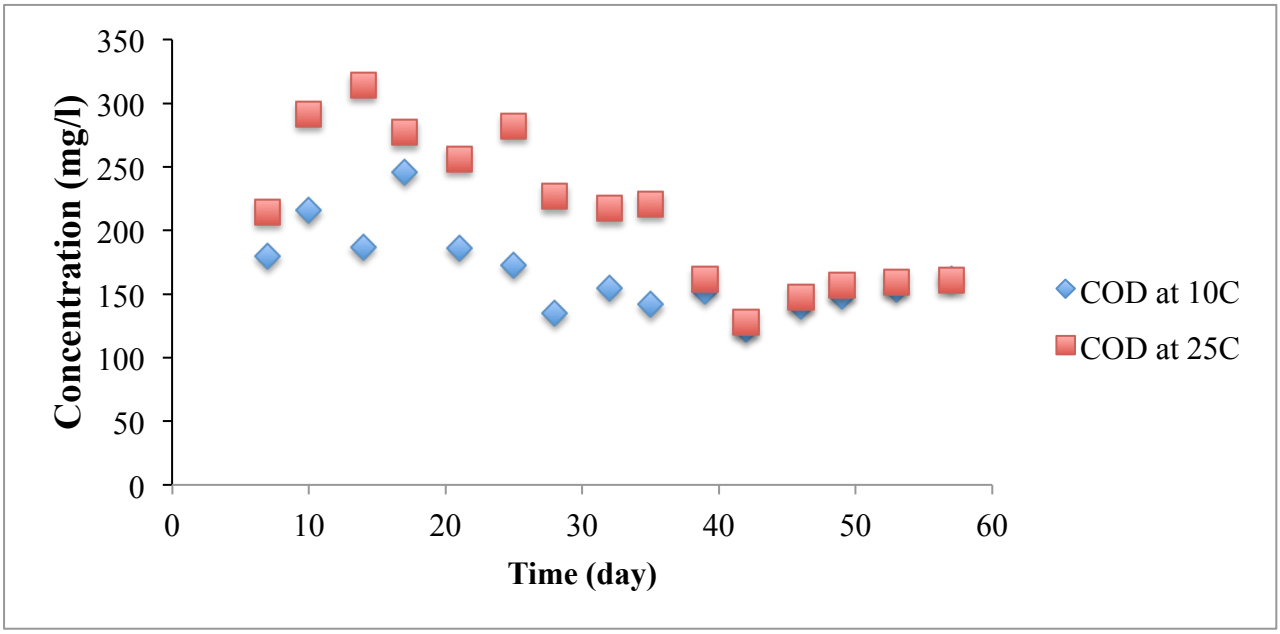

Figure 14: Variation of chemical oxygen demand (COD) value at the two incubation temperatures

4.4. Nitrification rate:

Figure 15 and 16 shows the typical curves obtained during measurements aiming to determine nitrification rate. As expected, the results illustrate a lag phase where there is no consumption of ammonia, followed by an exponential phase where ammonia is consumed at an increasing rate until it reaches a stationary concentration (around 1 mg/l at 25°C and around 0.2 mg/l at 10°C). Nitrification rate was calculated from the data representing the exponential phase. At 25°C, nitrification occurred faster than at 10°C. We can deduce from Figure 17 and 18 that the nitrification rate began approximately at 100 min at 25°C, whereas it took approximately 300 min at 10°C. Figure 17 and 18 represent the semi-logarithmic plots of nitrification derived using the exponential phase data of Figure 15 and 16. The equation of each linear regression is shown in each graph. The semi-logarithmic plots allowed quantitative comparison of how fast the ammonia in the wastewater was consumed and oxidized to first nitrite then to nitrate.

Table 13: Nitrification rate (min^{-1}) at both temperature derivated from the slopes

	25°C	**10°C**
$NH4^+$	-0.001 min^{-1}	-0.0023 min^{-1}
$NO2^-$	0.0053 min^{-1}	0.0027 min^{-1}
NO3-	0.0049 min^{-1}	0.0034 min^{-1}

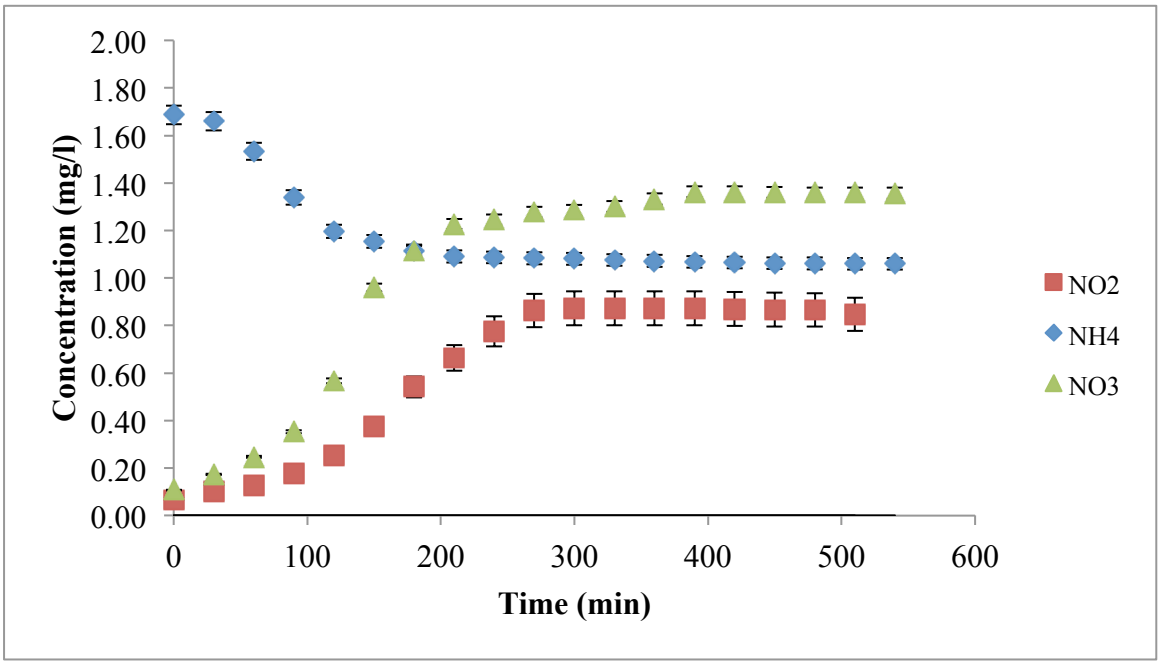

Figure 15: Nitrification rate at 25**°C.** Errors bars represent the standard deviation

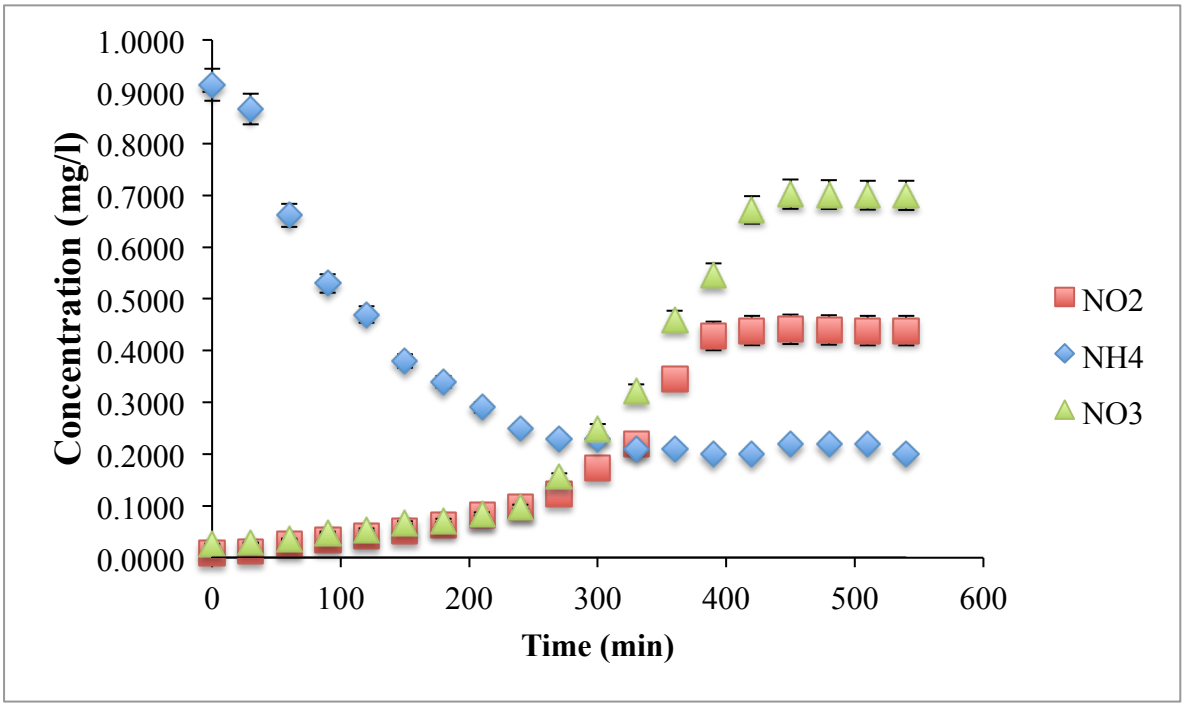

Figure 16: Nitrification rate at 10°C. Errors bars represent the standard deviation

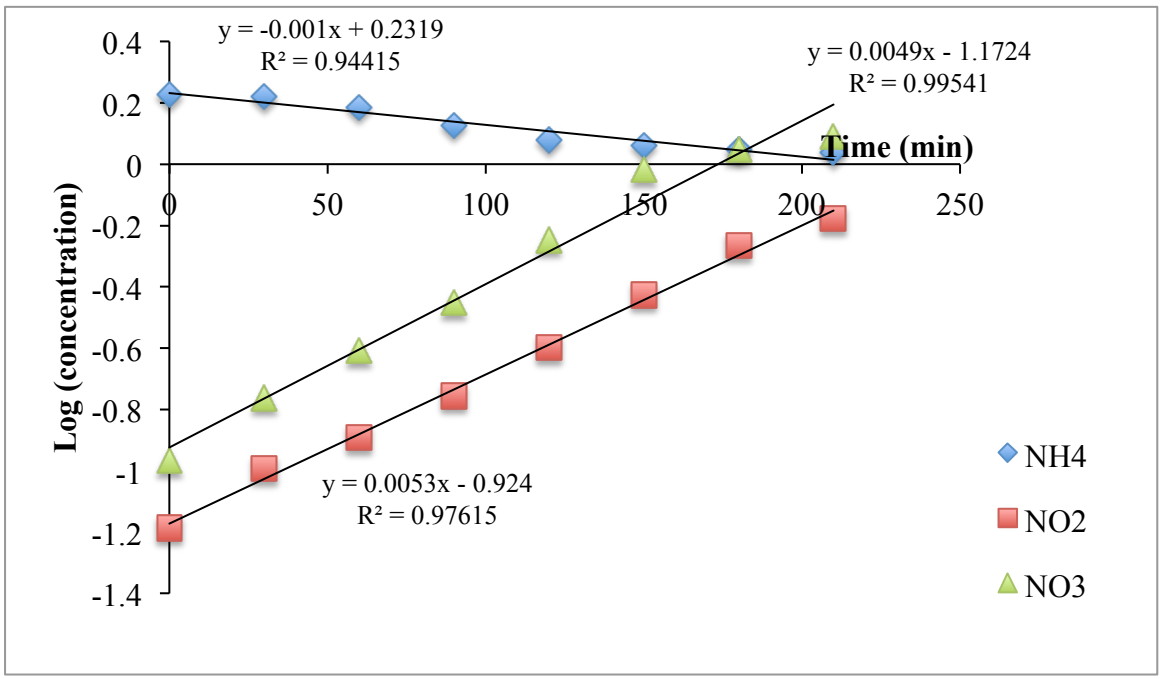

Figure 17: The semilogarithmic plots of nitrification based on spectrophotometric measurements at 25°C versus time incubation

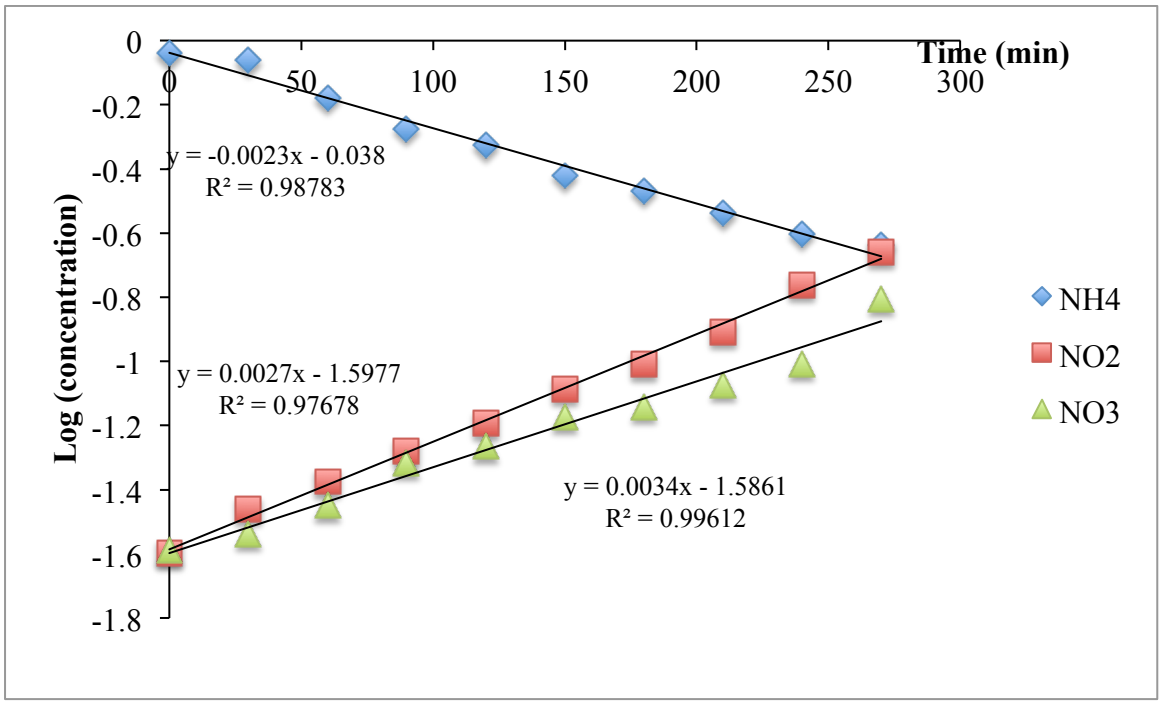

Figure 18: The semilogarithmic plots of nitrification based on spectrophotometric measurements at 10°C versus time incubation

4.5. MPN results:

MPN analysis was performed at time zero and at time 37 days in five replicates at both 10 and 25°C. The MPN tests were incubated for 60 days. Colony forming unit (CFU/ml) values were determined from the number of positive tubes based on the MPN table shown in Appendix A. The raw MPN results are summarized in Tables 14 and 15 for time zero and in Table 16 and 17 for the day 37 samples. In order to determine the concentration of bacteria present in the tubes, three sets of consecutives tubes were chosen, which expressed dilution of the organisms "to extinction". Therefore, at 25°C the combination of positive tubes results gave 3-1-0, and according to the MPN table, the MPN number of bacteria per one ml of the original undiluted sample would be $0.11x10^5$ CFU/ml. However, at 10°C, the combination of positives tubes gave 4-1-0, and the number of bacteria per one ml would be $0.17x10^4$ CFU/ml. The time zero sample incubated at 10°C had approximately 10 times lower concentration of nitrifiers than the time zero sample incubated at 25°C. For the day 37 samples, the amount of bacteria increased at both temperatures. The combination of positive tubes at 25°C gave 5-0-2, and according to the MPN table, the MPN number of bacteria per one ml would be $0.43x10^9$ CFU/ml. And at 10°C, the combination of positives tubes give 2-2-0 and the MPN number of bacteria per one ml would be $0.09x10^9$ CFU/ml. Again, the concentration of bacteria was higher at 25°C than those incubated at 10°C, however, this time only by a factor of approximately 5.

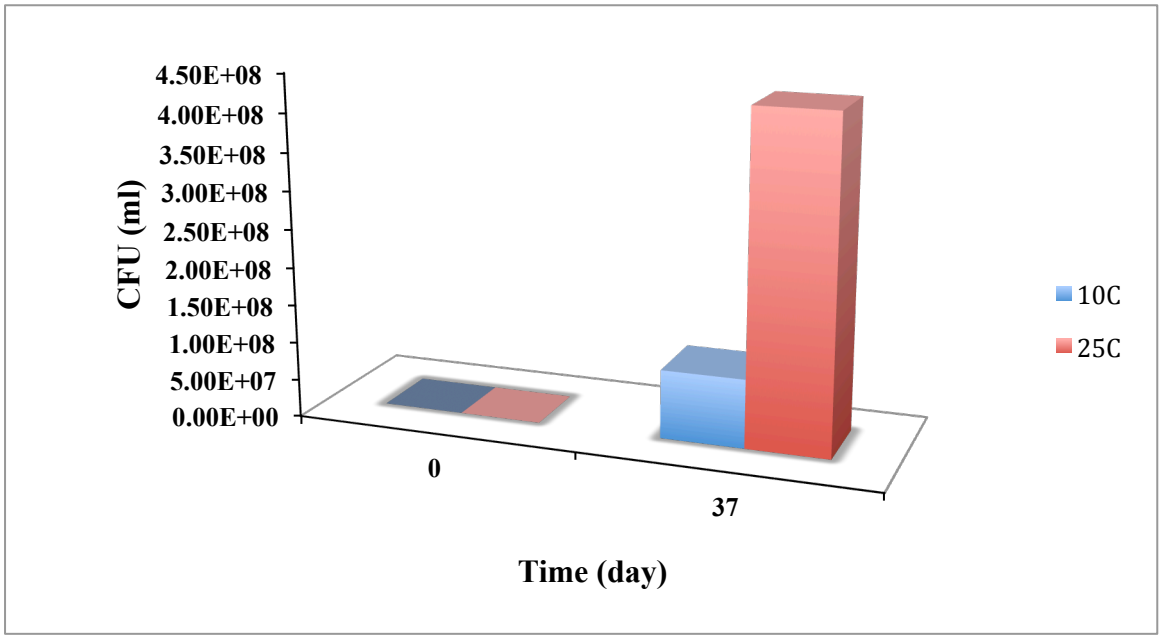

Figure 19: CFU of nitrifying bacteria

Table 14: MPN result at time zero incubated at 25°C

Dilutions	Number of positive tubes	Dilutions	Number of positive tubes
10^{-1}	0	10^{-6}	0
10^{-2}	0	10^{-7}	0
10^{-3}	1	10^{-8}	0
10^{-4}	3	10^{-9}	0
10^{-5}	1	10^{-10}	0

Table 15: MPN result at time zero incubated at 10°C

Dilutions	Number of positive tubes	Dilutions	Number of positive tubes
10^{-1}	2	10^{-6}	0
10^{-2}	2	10^{-7}	0
10^{-3}	4	10^{-8}	0
10^{-4}	1	10^{-9}	0
10^{-5}	0	10^{-10}	0

Table 16: MPN result at day 37 incubated at 25°C

Dilutions	Number of positive tubes	Dilutions	Number of positive tubes
10^{-1}	0	10^{-6}	0
10^{-2}	0	10^{-7}	3
10^{-3}	0	10^{-8}	5
10^{-4}	0	10^{-9}	0
10^{-5}	0	10^{-10}	2

Table 17: MPN result at day 37 incubated at 10°C

Dilutions	Number of positive tubes	Dilutions	Number of positive tubes
10^{-1}	5	10^{-6}	1
10^{-2}	0	10^{-7}	4
10^{-3}	0	10^{-8}	2
10^{-4}	4	10^{-9}	2
10^{-5}	2	10^{-10}	0

4.6. DGGE results:

Prior to DGGE analysis, DNA was extracted from the wastewater sample. The first gel electrophoresis showed that all samples contained DNA. For all DNA extracted, PCR amplification targeting bacterial 16S rRNA gene was performed. The gel electrophoresis of PCR products (Figure 20) showed that amplicons of the desired size were generated, except for sample 1 of time 23 days (T_{23}).

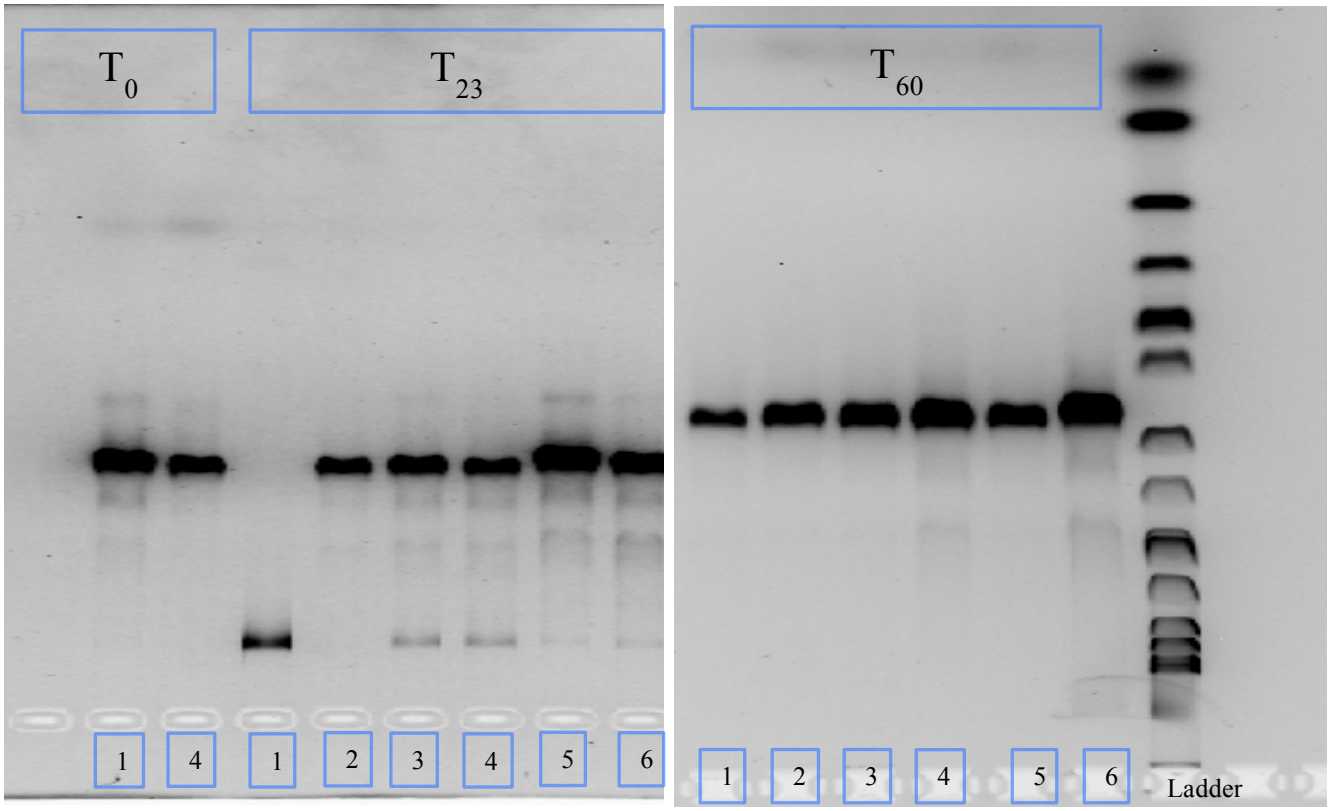

Figure 20: Section of agarose gel showing PCR products. The first two lines show amplicons generated from time zero sample (T_0), following by six lanes with time 23 days samples (T_{23}) and the last six lanes with time 60 days samples (T_{60}). The number 1, 2 and 3 are samples incubated at 25°C and 4, 5 and 6 are samples incubated at 10°C

The DGGE results are presented in Figure 21. In order to interpret the change in the bacterial community composition band patterns were compared. At time zero the two microbial communities appeared to be identical as the same band pattern was found at both temperatures. At time 23, no bands were observed for the sample number 1 as expected, based on amplicons of unexpected size following the PCR reaction (Figure 20). However, the remaining samples showed that the band pattern was similar for those, which were incubated at the same temperature. Some differences between the two temperatures could be observed, however, there was also some variation among replicate samples. At the end of the experiment (after 60 days), the difference in microbial community composition between

the two different temperatures became clear and banding pattern was identical in all three replicates.

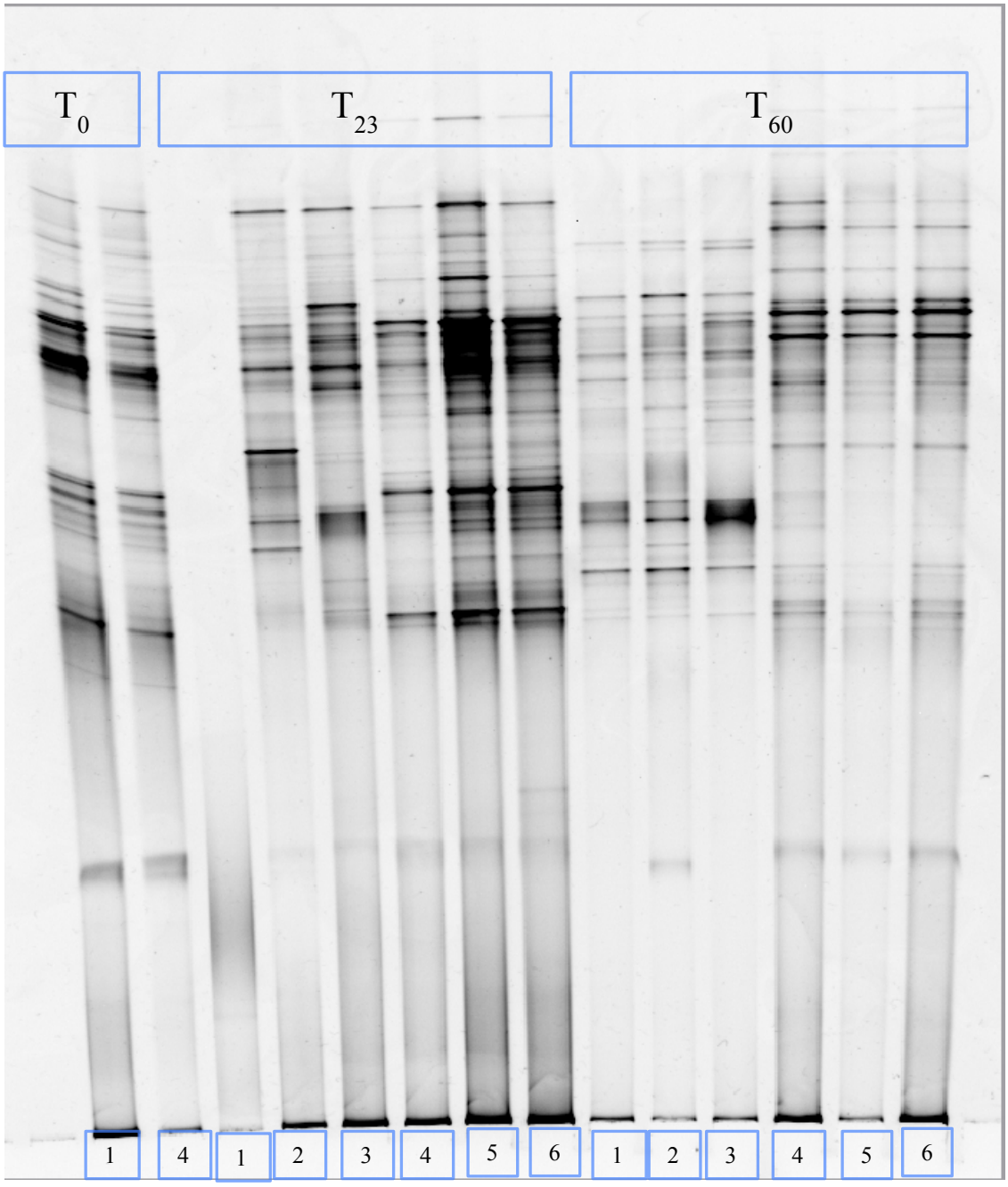

Figure 21: DGGE results. Amplification of extracted DNA with primers targeting 16S rRNA sequence of microbial DNA. The first two show time zero sample (T_0), following by six lanes with time 23 days samples (T_{23}) and the last six lanes with time 60 days samples (T_{60}). The number 1, 2 and 3 are samples incubated at 25°C and 4, 5 and 6 are samples incubated at 10°C

V. Discussions:

This section will discuss about the temperature and pH effect on nitrification process in wastewater. It will present also the change in diversity of nitrifying bacteria incubated at different temperatures through DGGE analysis and MPN method.

5.1. Ammonia, nitrite and nitrate measurement:

According to the results in Figure 8, 9, 10 and 11 the ammonia consumption will be at the minimum (approximately 1 mg/l) when the concentration of the feed will be exhausted. Therefore, if ammonia concentration is higher, its degradation will required a long time assuming the same biomass in the SBR. It was also found out that the concentrations of nitrite and nitrate increased as the ammonia was consumed in both reactors and their concentration would decline by the time of the next feeding. The increasing nitrite and nitrate concentration clearly confirms the activity of AOB, which convert ammonia to nitrite and nitrate under aerobic condition. On the other hand, in both temperatures, nitrate concentration increased while nitrite concentration is slightly increased. During these experiment, we suspected that DO concentration in the SRB was limited due to oxidation of ammonia to nitrite and AOB requires a high DO concentration than NOB. The change in concentration of ammonia, nitrite and nitrate might be due to the clogging of the air pumps, decrease in pH value or inaccuracy of DO concentration. These factors hampered the stability of ammonia, nitrite and nitrate concentration in the SBR. According to the figure 8 and 9, nitrification can occur at both temperatures 10 and 25°C. Several studies have been indicating that nitrification might be affected by temperature. However, according to the figure 9, any effect of temperature on nitrification is observed. The nitrification occur properly, but the ammonia, nitrite and nitrate concentration are slightly low compared to those incubated at 25°C. Jeyanayagam (2005) mentioned in his research that a decrease of temperature between 20 to 10°C will reduce the nitrification rate to 15%, which indicates that the nitrification is not strongly depended on the temperature. The temperature effect on nitrification is not truly clear, and no simple deduction could be drawn since the nitrification process might be associated to others parameters such DO, BOD and pH (Wang & Yang, 2002). Furthermore, aside of temperature effect, the change in pH has also an important influence on nitrification. All along this experiment, the pH has been checked and adjusted to 7.5 daily, if not the nitrification will be inhibited at pH below 4 and above 9.5 (Gerardi, 2003). For that, the pH has been maintained to be an alkaline pH equal to 7.5.

5.2. Sludge properties and COD:

TSS and VSS analysis are both very sensitive for precise measurement of the sample volume and other practical factors (e.g. careful handling of the filter), which can bias the amount of solids being transferred to the filter. The variation in TSS and VSS concentration (large error bars) observed during

this project might have been caused by such practical errors while doing the analysis. For example, the sample could not be thoroughly homogenized and mixed while taking the sample from the SBR. Also, the quantity of sample taken for each analysis could not be accurate and could affect the result. Low temperature affects not only nitrification or microbial growth but also the physicochemical properties of the sludge. As a result, the change in temperature has been associated to deterioration of organic matter and will reduce the composition of the sample (Arévalo, Ruiz, Pérez, & Gómez, 2014). This could explain why the concentration of TSS and VSS at 10°C is slightly lower than those analyzed at 25°C. Chemical Oxygen Demand (COD) is the amount of oxygen necessary to completely oxidize organic carbon in a given sample. According to the result on Figure 14, the COD concentration is lower at 10°C than at 25°C until approximately day 40, when the concentrations became nearly identical.

5.3. Nitrification rate:

Figure 15 and 16 showed the stages characteristic of a growth curve: lag phase, exponential phase, stationary phase and decline phase. Since we focus for the development of nitrification over time, we could not include the last stages due to lack of time. The lag phase showed no ammonium consumption. During this stage, the ammonium concentration is still at their higher concentration since the concentration of the feed is still sufficient. In the second step the exponential phase, a decline of ammonium concentration is observed at both temperatures. This decline showed the beginning of ammonium consumption over time until the concentration of the feed will be limited. However, in the exponential phase, a rapid accumulation of nitrite and nitrate was observed due to ammonia oxidation. Approximately 100 min, the ammonium concentration began to decline and nitrite accumulation started to increase at 25°C whereas it took approximately 300 min at 10°C. After 300 min at 25°C, the concentration of the three nitrogen forms became constant, reaching the stationary phase while at 10°C the stationary phase was reached at approximately 450 min. The midpoint of the nitrification curve represented the maximum rate of nitrification. As indicated from the Figure 15 and 16, the initial phase of the curve is approximately exponential. Then, in the stationary phase where the feed concentration is saturated, the ammonium concentration reached approximately 1 mg/l and the nitrite and nitrate concentration became constant. The asymptotically curve represented the value of nitrate production from the conversion of ammonia to nitrite, which in turn will oxidize to nitrate. Nitrification rates were obtained by measuring ammonia, nitrite and nitrate concentration over a period of 550 min starting from right after feeding the SBRs. Ammonia concentration reached minimum already after 200 min at 25°C, while it took approximately 300 min at 10°C. the calculated nitrification rate also clearly showed that the process occurred faster at higher temperature, despite the fact, that the low temperature SBR was adapted to 10°C for over a 60 day period of time by then. Based on this

comparison, 20°C appears to be the optimal temperature for nitrification.

Several studies have yielded different optimal temperatures, generally showing that the activity and the growth of nitrifying bacteria tend to increase with increasing temperature. It is difficult to quantify the temperature effect on nitrifier growth (Metcalf and Eddy, 1991a) as the optimal temperature and the pH are not fixed and depends on the concentration of ammonia present in the sample too (Quinlan, 1984). As the growth rate increased with increasing temperature, the trend of optimal temperature has been found around 35°C where the overall range is between 4to 50°C (Antoniou *et al.*, 1990 citing Loveless and Painter, 1968; Barnes and Bliss, 1983 citing Painter, 1970 and Focht and Chang, 1975; Churchwell *et al.*, 1980 citing Nelson, 1931 and Deppe and Engel, 1960). At 15°C, the activity of nitrifier bacteria has been stated to be low (Charley *et al.*, 1980), and increase when the temperature increased up to 30°C, and will slow down when the temperature is outside of 15°C (Fdz-Polanco *et al.*, 1994; Groeneweg *et al.*, 1994; Wild *et al.*, 1971). At lower temperatures, nitrification process seems to be affected but still the nitrification occurred (Azevedo *et al.*, 1995). Hence, other factors should be reconsidering when considering the links between temperature and the nitrification process (Fdz-Polanco *et al.*, 1994).

5.4. Microbial community analysis:

As described in the background section, the MPN method consists of the analysis of presence or absence of growing bacteria in the sample. In this instance, the MPN method presents some disadvantages. This method did not detect the loss of AOB or NOB during the incubation time but only the concentration of bacteria growing up to 60 days. This method indicates only the presence of active bacteria. The media used in the MPN tubes was not completely selective, hence it was difficult to avoid the outgrowth of nitrifiers by other heterotrophic bacteria (Watkins, 2011). As nitrifiers grow slowly, they need up to 80 days to for MPN incubations. The 60 days incubation time used in this project might have been too short for them. The change in color from blue to yellow and the production of gas in test tubes represented a positive result. The positive test tubes indicated the presence of nitrate, which was produced by AOB and those with negative results, indicated nitrate consumed during the denitrification process. That is why the sample did not turn to pink color. However, the MPN method was used to enumerate the number of bacteria in sample by observing whether or not the number of nitrifying bacteria will increase during the incubation. And from the results summarized in the Table 14, 15, 16 and 17, the amount of nitrifying bacteria in the wastewater increased over 60 days of incubation at both temperatures.

AOB and NOB preformed the nitrification process in wastewater. Therefore, while studying the nitrification rate in activated sludge, the microbial diversity of these organisms has been analyzed through PCR and DGGE. According to several studies, specific nitrifying bacteria has significant

impact during nitrification process (H. Li, Chen, Mu, & Gu, 2010). To target the microbial diversity of nitrifiers, DGGE and PCR technique were used. It is difficult to obtain a full representation of the nitrifier diversity of the sample by trusting solely on conventional methods. Molecular methodology such as PCR and DGGE were used during the experiment to overcome this problem. These methods help to reveal the nitrifying community from different temperatures (10 and 25°C). The application of PCR amplification provides us a great clarification of the nitrifier community in detail (Sunday & Seun, 2011). A number of researches have been assessed the microbiology of nitrifying bacteria in wastewater (B. Zhang, Sun, Ji, Liu, & Liu, 2010). Okabe et al, (1999) have discussed whether AOB (*Nitrosomonas*) or NOB (*Nitrospira*) is the main species present in the wastewater. Some researchers have been identified that the wastewater has been dominated by *Nitrosomonas* (Gieseke et al., 2001).

Unfortunately during this experiment, we could not identify the microbial distinction of AOB and NOB; we just observe the change in community incubated up to 60 days. The microbial community analysis was focused on the diversity of nitrifying bacteria. DGGE results showed that change in microbial population composition occurred at both temperatures revealing that nitrifier communities were distinctive from each other at 10 and 25°C. The DGGE result also indicated that there is a continuous change of microbial population over time, since the intensity of band pattern changed over time from time 0 to 60 days. The change in nitrifying communities might be explained by the temperature preference and the incubation time. The results obtained from the DGGE are comparable with what was found by other researchers, who showed similar change in microbial population diversity. The numbers of bands in each sample indicate the diversity of bacteria and the relative intensity of each band represent the relative abundance of the corresponding microorganism. The increase of band intensity indicated bacteria growing in abundance during the incubation time. Some differences in intensity of the bands have been observed also in relation with the temperature. The samples incubated at 25°C present more microbial diversity than those incubated at 10°C.

DGGE analysis provides valuable results when estimating the change in microbial diversity of sample. Despite of that, this method has some disadvantages. For example, it is important to be conscious about biases introduced during the PCR step (Jackson, Roden, & Churchill, 2000). Another limitation is the utilization of universal primers, which may restrain the amplification of 16S rRNA and again introduce bias. Due to the non-universality of universal primers, species diversity could be underestimated (Vallaeys et al., 1997). Generally, one band is assumed to correspond to one amplicon sequence that belongs to a defined specie (de Araujo & Schneider, 2008). However, since the amplification of 16S rRNA gene fragments may generate multiple bands from the same species, a false increase of the number of bands can be observed (Schmalenberger, Schwieger, & Tebbe, 2001). Multiple bands belonging to the same species could also be derived from the denaturation of the

amplicons or from two versions of 16S rRNA genes existing in a define organism (de Araujo & Schneider, 2008). In practice, it is impossible to get an exact representation of microbial community composition when using DGGE.

VI. Conclusion

Overall, this project provided valuable insights into the dynamics of the development of a mixed nitrifier culture, active at as low temperature as 10°C. the results showed that 60 days of SBR operation was long enough to achieve stable nitrification activity at both incubation temperatures (10 and 25°C). The different incubation temperatures have not appeared to affect the nitrification process in a sense that nitrification was ongoing at both 10 and 25°C. Hence, we found that 10°C was not sufficiently cold to inhibit the nitrification process. Also, even higher nitrification rates could probably have been achieved with increasing the temperature above 25°C. The nitrifying bacteria will take long time to acclimate to new environmental conditions if the wastewater temperature changes. Aside of impact to the growth of bacteria, the change in temperature affect also the sludge properties such the suspend solids or the COD. These groups by for example sequencing were not possible in this project. However, the change in bacterial community composition was clearly shown by PCR-DGGE analysis.
Nitrifying population performing nitrification was analyzed through culture-based method, i.e., MPN and molecular tools i.e., PCR and DGGE. The MPN method to enumerate nitrifying bacteria was successful as a comparative approach to investigate the number of organisms, and showed that the number of nitrifiers was approximately 5 times higher at 25°C compared to 10°C after 37 days of SBR operation. The results of the fingerprinting (DGGE) method suggested that composition of the developed mixed culture was distinct at the two different incubation temperatures. Hence, we can assume that a more cold-adapted community was formed under the experimental conditions chosen.
The conclusions deducted from this experiment are:

- The temperature of 10°C did not inhibit the growth of nitrifying bacterial community.
- Temperature did influence the observed nitrification rate after 60 days of SBR operation.
- Concentration of nitrifying bacteria increased over time at both temperatures, but remained lower at 10°C compared to 25°C.
- A change in bacterial community composition has been observed over the 60 days of incubation time.
- Clearly distinct communities evolved at the two different incubation temperatures.

For future studies, aiming at better understanding of nitrification process in SBR, we recommend that:

- The investigation of denitrification performance should be included.
- The investigation of effect of pH in both nitrification and bacterial growth should be

performed.

- Other important parameters, such as alkalinity and SRT and DO concentration should also be investigated. Identification of nitrifying organisms using sequencing and culture-based methods should be included in addition to analysis of the bacterial diversity.

References

Amann, R. I., Ludwig, W., & Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews, 59*(1), 143-169.

Amini, M., Pourhossein, M., & Talebi, M. (2005). A chemiluminescence flow injection system for nitrite ion determination. *Journal of the Iranian Chemical Society, 2*(4), 305-314.

Andrews, J. H., & Harris, R. F. (1986). r-and K-selection and microbial ecology *Advances in microbial ecology* (pp. 99-147): Springer.

Anthonisen, A., Loehr, R., Prakasam, T., & Srinath, E. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control Federation)*, 835-852.

APHA. (1981). *Standard Methods for the Examination of Water and Wastewater 15ed*: APHA American Public Health Association.

Arévalo, J., Ruiz, L., Pérez, J., & Gómez, M. (2014). Effect of temperature on membrane bioreactor performance working with high hydraulic and sludge retention time. *Biochemical Engineering Journal, 88*, 42-49.

Arp, D. J., Sayavedra-Soto, L. A., & Hommes, N. G. (2002). Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. *Archives of Microbiology, 178*(4), 250-255.

Belser, L. W. (1979). Population ecology of nitrifying bacteria. *Annual reviews in microbiology, 33*(1), 309-333.

Benefield, L. D., & Randall, C. W. (1981). Biological process design for wastewater treatment *Prentice Hall Series in Environmental Sciences*: Prentice hall.

Bock, E., Koops, H.-P., Ahlers, B., Harms, H., Balows, A., Truper, H., . . . Schleifer, K. (1992). Oxidation of inorganic nitrogen compounds as energy source. *The Prokaryotes.*(Ed. 2), 414-430.

Bothe, H., Jost, G., Schloter, M., Ward, B. B., & Witzel, K.-P. (2000). Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microbiology Reviews, 24*(5), 673-690.

Brakstad, O. G., & Bonaunet, K. (2006). Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0–5 C) and bacterial communities associated with degradation. *Biodegradation, 17*(1), 71-82.

Brochier-Armanet, C., Boussau, B., Gribaldo, S., & Forterre, P. (2008). Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nature Reviews Microbiology, 6*(3), 245-252.

Burrell, P., Keller, J., & Blackall, L. L. (1999). Characterisation of the bacterial consortium involved in nitrite oxidation in activated sludge. *Water Science and Technology, 39*(6), 45-52.

Chang, S. W., Hyman, M. R., & Williamson, K. J. (2002). Cooxidation of naphthalene and other polycyclic aromatic hydrocarbons by the nitrifying bacterium, Nitrosomonas europaea. *Biodegradation, 13*(6), 373-381.

Charley, R., Hooper, D., & McLee, A. (1980). Nitrification kinetics in activated sludge at various temperatures and dissolved oxygen concentrations. *Water Research, 14*(10), 1387-1396.

Cheremisinoff, N. (1996). Biological Degradation of hazardous Waste. *Biotechnology for Waste and Wastewater Treatment. Noyes Publications: Westwood, New Jersey, USA*, p37-110.

Curtis, T., & Sloan, W. (2006). Towards the design of diversity: stochastic models for community assembly in wastewater treatment plants. *Water Science & Technology, 54*(1), 227-236.
Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K.-H., & Wagner, M. (2001). In Situ Characterization ofNitrospira-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. *Applied and Environmental Microbiology, 67*(11), 5273-5284.
Dangcong, P., Bernet, N., Delgenes, J.-P., & Moletta, R. (2000). Effects of oxygen supply methods on the performance of a sequencing batch reactor for high ammonium nitrification. *Water environment research*, 195-200.
De Beer, D., & Schramm, A. (1998). Identification and activities in situ of Nitrosospira and Nitrospira spp. as dominant populations. *Applied and Environmental Microbiology, 64*(9), 3480-3485.
De Renzo, D. J. (1978). *Nitrogen control and phosphorus removal in sewage treatment*: Noyes Data Corp.
Eilersen, A. M., Henze, M., & Kløft, L. (1994). Effect of volatile fatty acids and trimethylamine on nitrification in activated sludge. *Water Research, 28*(6), 1329-1336.
EPA, U. (1993). Process design manual of nitrogen control. *EP A.[Links]*.
Erguder, T. H., Boon, N., Wittebolle, L., Marzorati, M., & Verstraete, W. (2009). Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. *FEMS Microbiology Reviews, 33*(5), 855-869.
Forgie, D. (1988). *Development of a leachate treatment selection model.* Paper presented at the ASCE Environ. Eng. Natl. Conf.
Fraga, D., Meulia, T., & Fenster, S. (2008). Current protocols: essential laboratory techniques: John Wiley & Sons: Hoboken, NJ.
Furukawa, K., Ike, A., & Fujita, M. (1993). Preparation of marine nitrifying sludge. *Journal of fermentation and bioengineering, 76*(2), 134-139.
Ge, S., Jiang, Y., & Wei, S. (2015). Gross Nitrification Rates and Nitrous Oxide Emissions in an Apple Orchard Soil in Northeast China. *Pedosphere, 25*(4), 622-630. doi:http://dx.doi.org/10.1016/S1002-0160(15)30042-4
Ge, S. J., Wang, S. Y., Yang, X., Qiu, S., Li, B. K., & Peng, Y. Z. (2015). Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review. *Chemosphere, 140*, 85-98. doi:10.1016/j.chemosphere.2015.02.004
Gerardi, M. H. (2003). *Nitrification and denitrification in the activated sludge process*: John Wiley & Sons.
Gieseke, A., Purkhold, U., Wagner, M., Amann, R., & Schramm, A. (2001). Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. *Applied and Environmental Microbiology, 67*(3), 1351-1362.
Grady, L., Daigger, C., & Lim, G. (1999). Biological wastewater treatment: Marcel Dekker, Inc. New York.
Gray, N. F. (1989). *Biology of waste water treatment*: Oxford University Press.
Grüntzig, V., Stres, B., del Rio, H. A., & Tiedje, J. M. (2002). *Improved protocol for T-RFLP by capillary electrophoresis*: Michigan State University, Center for Microbial Ecology.
Gupta, S., & Sharma, R. (1996). Biological oxidation of high strength nitrogenous wastewater. *Water Research, 30*(3), 593-600.
Hanaki, K., Wantawin, C., & Ohgaki, S. (1990). Effects of the activity of heterotrophs on nitrification in a suspended-growth reactor. *Water Research, 24*(3), 289-296.

Hatzenpichler, R., Lebedeva, E. V., Spieck, E., Stoecker, K., Richter, A., Daims, H., & Wagner, M. (2008). A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proceedings of the National Academy of Sciences, 105*(6), 2134-2139.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome research, 6*(10), 986-994.

Higuchi, K., & Motomizu, S. (1999). Flow-Injection Spectrophotometric Determination of Nitrite and Nitrate in Biological Samples. *Analytical sciences, 15*(2), 129-134.

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology, 11*, 1026-1030.

Hovanec, T. A., Taylor, L. T., Blakis, A., & Delong, E. F. (1998). Nitrospira-like bacteria associated with nitrite oxidation in freshwater aquaria. *Applied and Environmental Microbiology, 64*(1), 258-264.

Ichihashi, O., Satoh, H., & Mino, T. (2006). Effect of soluble microbial products on microbial metabolisms related to nutrient removal. *Water Research, 40*(8), 1627-1633.

Isaka, K., Yoshie, S., Sumino, T., Inamori, Y., & Tsuneda, S. (2007). Nitrification of landfill leachate using immobilized nitrifying bacteria at low temperatures. *Biochemical Engineering Journal, 37*(1), 49-55.

Jeyanayagam, S. (2005). True confessions of the biological nutrient removal process. *Florida Water Resources Journal, 1*, 37-46.

Jin, T., Zhang, T., & Yan, Q. (2010). Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR. *Applied microbiology and biotechnology, 87*(3), 1167-1176.

Junier, P., Molina, V., Dorador, C., Hadas, O., Kim, O.-S., Junier, T., . . . Imhoff, J. F. (2010). Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. *Applied microbiology and biotechnology, 85*(3), 425-440.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., & Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology, 64*(8), 3042-3051.

Keener, W. K., & Arp, D. J. (1993). Kinetic studies of ammonia monooxygenase inhibition in Nitrosomonas europaea by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. *Applied and Environmental Microbiology, 59*(8), 2501-2510.

Könneke, M., Bernhard, A. E., José, R., Walker, C. B., Waterbury, J. B., & Stahl, D. A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature, 437*(7058), 543-546.

Kuenen, J. G. (2008). Anammox bacteria: from discovery to application. *Nature Reviews Microbiology, 6*(4), 320-326.

Li, H., Chen, S., Mu, B.-Z., & Gu, J.-D. (2010). Molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in high-temperature petroleum reservoirs. *Microbial ecology, 60*(4), 771-783.

Li, H. B., Zhou, B. H., Tian, Z. Y., Song, Y. H., Xiang, L. C., Wang, S. Y., & Sun, C. (2015). Efficient biological nitrogen removal by Johannesburg-Sulfur autotrophic denitrification from low COD/TN ratio municipal wastewater at low temperature. *Environmental Earth Sciences, 73*(9), 5027-5035. doi:10.1007/s12665-015-4231-z

Madigan, M. T., Martinko, J. M., Parker, J., & Brock, T. D. (1997). *Biology of microorganisms* (Vol. 985): prentice hall Upper Saddle River, NJ.

Madoni, P., Davoli, D., & Guglielmi, L. (1999). Response of SOUR and AUR to heavy metal contamination in activated sludge. *Water Research, 33*(10), 2459-2464.

Martens-Habbena, W., Berube, P. M., Urakawa, H., José, R., & Stahl, D. A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature, 461*(7266), 976-979.

McCarty, G. (1999). Modes of action of nitrification inhibitors. *Biology and Fertility of Soils, 29*(1), 1-9.

McClintock, S. A., Randall, C. W., & Pattarkine, V. M. (1993). Effects of temperature and mean cell residence time on biological nutrient removal processes. *Water environment research*, 110-118.

Mincer, T. J., Church, M. J., Taylor, L. T., Preston, C., Karl, D. M., & DeLong, E. F. (2007). Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environmental microbiology, 9*(5), 1162-1175.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E., & Stahl, D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology, 62*(6), 2156-2162.

Moter, A., & Göbel, U. B. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *Journal of microbiological methods, 41*(2), 85-112.

Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology, 59*(3), 695-700.

Okabe, S., Satoh, H., & Watanabe, Y. (1999). In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Applied and Environmental Microbiology, 65*(7), 3182-3191.

Park, S.-J., Oh, J.-W., & Yoon, T.-I. (2003). The role of powdered zeolite and activated carbon carriers on nitrification in activated sludge with inhibitory materials. *Process Biochemistry, 39*(2), 211-219.

Poduska, R. A., & Andrews, J. F. (1975). Dynamics of nitrification in the activated sludge process. *Journal (Water Pollution Control Federation)*, 2599-2619.

Preston, C. M., Wu, K. Y., Molinski, T. F., & DeLong, E. F. (1996). A psychrophilic crenarchaeon inhabits a marine sponge: Cenarchaeum symbiosum gen. nov., sp. nov. *Proceedings of the National Academy of Sciences, 93*(13), 6241-6246.

Prosser, I. (1989). Autotrophic nitrification in bacteria. *Adv. Microb. Physiol, 20*, 125-192.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H.-P., & Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Applied and Environmental Microbiology, 66*(12), 5368-5382.

Qader, H. A. (2013). Spectrophotometric Determination of Nitrite in Curing Meat Samples.

Quinlan, A. V. (1984). Prediction of the optimum pH for ammonia-n oxidation by Nitrosomonas europaea in well-aerated natural and domestic-waste waters. *Water Research, 18*(5), 561-566.

Ragsdale, F. (2013). Wastewater system operator's manual. *Ragsdale and Associates Training Specialists, Omaha Nebraska, NE*.

Rasche, M., Hicks, R., Hyman, M., & Arp, D. (1990). Oxidation of monohalogenated ethanes and n-chlorinated alkanes by whole cells of Nitrosomonas europaea. *Journal of bacteriology, 172*(9), 5368-5373.

Rastogi, G., & Sani, R. K. (2011). Molecular techniques to assess microbial community structure, function, and dynamics in the environment *Microbes and microbial technology* (pp. 29-57): Springer.

Rittmann, B. E., & McCarty, P. L. (2012). *Environmental biotechnology: principles and applications*: Tata McGraw-Hill Education.

Rodriguez Caballero, A. (2011). STUDY OF BACTERIAL COMMUNITIES:–A WASTEWATER TREATMENT PERSPECTIVE.

Rogers, G. L. (1983). *Nitrifier populations and kinetics in selected aquaculture water reuse biofilters.* Colorado State University.

Rowan, A. K., Snape, J. R., Fearnside, D., Barer, M. R., Curtis, T. P., & Head, I. M. (2003). Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS microbiology ecology, 43*(2), 195-206.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., . . . Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science, 239*(4839), 487-491.

Schleper, C., & Nicol, G. W. (2010). Ammonia-oxidising archaea—physiology, ecology and evolution. *Advances in microbial physiology, 57*, 1-41.

Schmid, M. C., Maas, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B., . . . Kuenen, J. G. (2005). Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Applied and Environmental Microbiology, 71*(4), 1677-1684.

Sedlak, R. I. (1991). *Phosphorus and nitrogen removal from municipal wastewater: principles and practice*: CRC press.

Seviour, R. J., & Blackall, L. (2012). *The microbiology of activated sludge*: Springer Science & Business Media.

Siripong, S., & Rittmann, B. E. (2007). Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. *Water Research, 41*(5), 1110-1120. doi:http://dx.doi.org/10.1016/j.watres.2006.11.050

Smith, C. J., & Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS microbiology ecology, 67*(1), 6-20.

Spieck, E., & Bock, E. (2005). The lithoautotrophic nitrite-oxidizing bacteria *Bergey's Manual® of Systematic Bacteriology* (pp. 149-153): Springer.

Strous, M., & Jetten, M. S. (2004). Anaerobic oxidation of methane and ammonium. *Annu. Rev. Microbiol., 58*, 99-117.

Sun, J., Zhang, X., Broderick, M., & Fein, H. (2003). Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors, 3*(8), 276-284.

Sunday, A. O., & Seun, A. O. (2011). Biological wastewater treatment: microbiology, chemistry, and diversity measurement of ammonia oxidizing bacteria. *African Journal of Microbiology Research, 5*(32), 5831-5840.

Suwa, Y., Imamura, Y., Suzuki, T., Tashiro, T., & Urushigawa, Y. (1994). Ammonia-oxidizing bacteria with different sensitivities to (NH 4) 2 SO 4 in activated sludges. *Water Research, 28*(7), 1523-1532.

Taylor, J. R. (1982). An introduction to error analysis.

Terada, A., Zhou, S., & Hosomi, M. (2011). Presence and detection of anaerobic ammonium-oxidizing (anammox) bacteria and appraisal of anammox process for high-strength nitrogenous wastewater treatment: a review. *Clean Technologies and Environmental Policy, 13*(6), 759-781.

Tokutomi, T. (2004). Operation of a nitrite-type airlift reactor at low DO concentration. *Water Science & Technology, 49*(5-6), 81-88.

Tomiyasu, T., Konagayoshi, Y., Anazawa, K., & Sakamoto, H. (2001). A kinetic method for the determination of nitrite by its catalytic effect on the oxidation of chlorpromazine with nitric acid. *Analytical sciences, 17*(12), 1437-1440.

Van Dongen, U., Jetten, M. S., & Van Loosdrecht, M. (2001). The SHARON®-Anammox® process for treatment of ammonium rich wastewater. *Water Science and Technology, 44*(1), 153-160.

Van Staden, J., & Makhafola, M. (1996). Spectrophotometric determination of nitrite in foodstuffs by flow injection analysis. *Fresenius' journal of analytical chemistry, 356*(1), 70-74.

Vikesland, P. J., Love, N. G., Chandran, K., Fiss, E. M., Rebodos, R., Zaklikowski, A. E., . . . Ferguson, B. (2007). *Seasonal chlorination practices and impacts to chloraminating utilities*: American Water Works Association.

Volcke, E., Van Loosdrecht, M., & Vanrolleghem, P. (2006). Controlling the nitrite: ammonium ratio in a SHARON reactor in view of its coupling with an Anammox process. *Instrumentation, Control and Automation for Water and Wastewater Treatment and Transport Systems IX, 53*(4), 45-54.

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N., & Daims, H. (2002). Microbial community composition and function in wastewater treatment plants. *Antonie van Leeuwenhoek, 81*(1-4), 665-680.

Wagner, M., Rath, G., Amann, R., Koops, H.-P., & Schleifer, K.-H. (1995). In situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology, 18*(2), 251-264.

Wahman, D. G., Kirisits, M. J., Katz, L. E., & Speitel, G. E. (2011). Ammonia-oxidizing bacteria in biofilters removing trihalomethanes are related to Nitrosomonas oligotropha. *Applied and Environmental Microbiology*.

Wang, R., & Yang, J. (2002). Industrial ecology. *Encyclopedia of Life Support Systems: Hazardous Waste Management*.

Watkins, S. (2011). *Physico-chemical and microbial factors affecting the operation of a package wastewater treatment plant*: University of Portsmouth.

Welander, U., Henrysson, T., & Welander, T. (1997). Nitrification of landfill leachate using suspended-carrier biofilm technology. *Water Research, 31*(9), 2351-2355.

Welander, U., Henrysson, T., & Welander, T. (1998). Biological nitrogen removal from municipal landfill leachate in a pilot scale suspended carrier biofilm process. *Water Research, 32*(5), 1564-1570.

Whalen, J. K., & Sampedro, L. (2010). *Soil ecology and management*: CABI.

Wild Jr, H. E., Sawyer, C. N., & McMahon, T. C. (1971). Factors affecting nitrification kinetics. *Journal (Water Pollution Control Federation)*, 1845-1854.

Wood, P. M. (1986). Nitrification as a bacterial energy source.

Yamagishi, T., Leite, J., Ueda, S., Yamaguchi, F., & Suwa, Y. (2001). Simultaneous removal of phenol and ammonia by an activated sludge process with cross-flow filtration. *Water Research, 35*(13), 3089-3096.

Ydstebø, L., Bilstad, T., & Barnard, J. (2000). Experience with biological nutrient removal at low temperatures. *Water environment research*, 444-454.

Zhang, B., Sun, B., Ji, M., Liu, H., & Liu, X. (2010). Quantification and comparison of ammonia-oxidizing bacterial communities in MBRs treating various types of wastewater. *Bioresource Technology, 101*(9), 3054-3059.

Zhang, S. M., Sha, C. Q., Jiang, W., Li, W. G., Zhang, D. Y., Li, J., . . . Piao, Y. J. (2015). Ammonium removal at low temperature by a newly isolated heterotrophic nitrifying and aerobic denitrifying bacterium Pseudomonas fluorescens wsw-1001. *Environmental Technology, 36*(19), 2488-2494. doi:10.1080/09593330.2015.1035759

Appendix: Five tubes MPN table
(source:http://www.jlindquist.net/generalmicro/102dil3a.html)

No. of Tubes Positive in			MPN in the inoculum of the middle set of tubes	No. of Tubes Positive in			MPN in the inoculum of the middle set of tubes
First set	Middle set	Last set		First set	Middle set	Last set	
0	0	0	‹0.01	4	3	0	0.27
0	0	1	0.02	4	3	1	0.33
0	1	0	0.02	4	4	0	0.34
0	2	0	0.04	5	0	0	0.23
1	0	0	0.02	5	0	1	0.31
1	0	1	0.04	5	0	2	0.43
1	1	0	0.04	5	1	0	0.33
1	1	1	0.06	5	1	1	0.46
1	2	0	0.06	5	1	2	0.63
2	0	0	0.05	5	2	0	0.49
2	0	1	0.07	5	2	1	0.7
2	1	0	0.07	5	2	2	0.94
2	1	1	0.09	5	3	0	0.79
2	2	0	0.09	5	3	1	1.1
2	3	0	0.12	5	3	2	1.4
3	0	0	0.08	5	3	3	1.8
3	0	1	0.11	5	4	0	1.3
3	1	0	0.11	5	4	1	1.7
3	1	1	0.14	5	4	2	2.2
3	2	0	0.14	5	4	3	2.8
3	2	1	0.17	5	4	4	3.5
4	0	0	0.13	5	5	0	2.4
4	0	1	0.17	5	5	1	3.5
4	1	0	0.17	5	5	2	5.4
4	1	1	0.21	5	5	3	9.2
4	1	2	0.26	5	5	4	16
4	2	0	0.22	5	5	5	›24
4	2	1	0.26				