Study of the Anaerobic Methane Oxidation Coupled to Nitrate Denitrification

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Applied Science

in

Civil Engineering

Waterloo, Ontario, Canada, 2013

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Methane can be a potentially inexpensive, widely available electron donor for biological denitrification of wastewater, landfill leachate or drinking water, while no studies have clearly shown nitrate reduction to nitrogen gas. Recently anaerobic methane oxidation (AMO) coupled to partial denitrification (nitrite to nitrogen gas) was found by several studies. A microbial consortium, enriched from anoxic sediments, oxidized methane to carbon dioxide coupled to denitrification in the complete absence of oxygen, though the rates and pathways of AMO coupled to denitrification are still poorly understood. In this study, direct AMO coupled to denitrificaiton of nitrate was proved to be possible and its kinetic parameters were experimentally determined. Using a set of batch experiments designed to provide the best estimates of each parameter, these parameters were obtained: maximum specific growth rate (μ_{max}) = 0.121/day, maximum substrate consumption rate (q_{max}) = 1.63 mg COD/mg cells-day, true yield (Y) = 0.074 mg cells/mg COD, half maximum-rate substrate concentration (K_s) = 85 μM CH_4, and endogenous decay rate (b) = 0.03/day. This study firstly characterized kinetic parameters of anaerobic methanotrophic denitrifiers, which will substantially help understand anaerobic methane oxidation in natural systems and accelerate methane-utilizing denitrification in engineering systems.

Acknowledgements

I would like sincerely thank my supervisor Dr. Hyung-Sool Lee for the guidance and active participation during this research. His encouragement and patience were a great help to me. I am very privileged to have spent two years learning from this well-respected expert.

I would like to thank all of my colleagues in our research group. I would like to express my sincere gratitude to Dr. Elsayed Elbeshbishy and Dr. Junyeong An for their enormous help with various experimental techniques and advice through valuable discussions related to my project. I also acknowledge the valuable support and encouragement of my colleagues Dr. Weiwei Du， Yaohuan Gao, Hyoengu Yeo, Mohammed Galib, Junyeong Sim, Bipro Ranjan Dhar.

I would like to appreciate staffs, technicians, and co-op students who provided invaluable help in my research.

I would like to thank Dr. Sheree Pagsuyoin and Dr. Mahesh Pandey for reviewing my thesis.

I would like to acknowledge the financial support received from University of Waterloo, the Natural Sciences and Engineering Research Council of Canada.

Lastly, I would like to acknowledge the endless love, encouragement and support of my friends and family. Most of all, I would like to thank my wife, Qiasosi Deng who always kept believing in me.

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List of Abbreviations

AMO	Anaerobic Methane Oxidation
b	Endogenous-decay coefficient
BOD	Biochemical oxygen demand
BNR	Biological Nutrient Removal
CH_4	Methane
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
ECD	Electron Capture Detector
GC	Gas Chromatography
HFMBR	hollow-fiber membrane-biofilm reactor
K_H	Henry's Law constant
K_s	Half saturation constant
N_2	Dinitrogen
NDAMO	Nitrite Dependent Anaerobic Methane Oxidization
NH_3-N	Ammonia Nitrogen
NO	Nitrogen oxide
N_2O	Nitrous oxide
NO_2-N	Nitrite Nitrogen
NO_3-N	Nitrate Nitrogen
RAS	Return Activated Sludge
S	Substrate
T	Temperature
TCD	Thermal Conductivity Detector
V_G	Headspace volume
V_L	Liquid volume
WW	Wastewater
WWTP	Wastewater Treatment Plant
X	Biomass
Y	Growth yield
μ_{max}	Maximum specific growth rate
q_{max}	Maximum specific rate of substrate

1. Introduction

1.1 Motivation

Methane (CH_4) is an important greenhouse gas, which so far has contributed about 20% of global warming. Atmospheric concentrations of methane have risen 2.6-fold since preindustrial times (Knittel and Boetius, 2009). After several years of stagnation, there was again a clear increase in global methane concentration in 2007 (Rigby , 2008).

Freshwater habitats like natural wetlands and rice fields are major source (38%) of atmospheric methane (Kumaraswamy et al., 2001). Microbially mediated anaerobic methane oxidation (AMO) is very important to Earth's climate because it consumes methane produced from natural sediments before it escapes to the atmosphere (DeLong, 2000). This process is estimated to oxidize up to 90% of methane produced in anaerobic marine sediments (Reeburgh et al., 1993). AMO has also been observed in freshwater environments, although its contribution as a methane sink has not been quantified (Eller et al., 2005). Despite extensive studies, only a few microorganisms capable of AMO have been isolated. Molecular and biogeochemical studies have shown that for marine environments the microorganisms responsible for AMO consist of a consortium of methanogen-related archaea, and sulfate-reducing bacteria (SRB) (Niemann et al., 2006). The nature of the cooperation between the archaea and bacteria has so not been elucidated. Environmental genomic studies of anaerobic methanotrophic archaea (ANME) support the hypothesis that ANME can oxidize methane via a reverse methanogenesis process to a substrate used by SRB (Hallam et al., 2004).

In contrast to the numerous studies about AMO coupled to sulfate reduction mentioned above, there were only a few publications that focus on the denitrifying anaerobic methane oxidation processes. Methane oxidation coupled to denitrification has so far received the most attention in the field of hydrogeology. Contamination with nitrate and nitrite occurs frequently in groundwater where electron donors are limiting. Methane plumes often form around landfills, and their attenuation has sometimes been attributed to denitrification (Tamura , 2007). Denitrification is the reduction of nitrate and nitrite to nitrous oxide and dinitrogen gas. Many bacteria and archaea have the potential to denitrify (Philippot, 2002), and numerous organic and inorganic compounds can be used as an electron donor for denitrification. Although methane is a thermodynamically favourable electron donor for both nitrate and nitrite reduction (-765 and -928 kJ mol^{-1} CH_4 respectively (Raghoebarsing et al., 2006), experimental evidence for its complete anaerobic oxidation was only found recently.

The first enrichment of a denitrifying anaerobic methane oxidation culture was reported by Raghoebarsing and colleagues (2006). The culture was obtained from freshwater canal sediments after 16 months anaerobic incubation in the presence of methane, nitrate and nitrite. Two groups of microorganisms were found to dominate the culture, namely a bacterium (80% of the microbial population) belonging to the NC10 division, and an archaeon (10% of the microbial population) that was distantly related to anaerobic methanotrophic archaea, the culture was operated at 25°C. Later, the archaeal population declined and disappeared from the culture, indicating that the enriched bacteria alone may be able to couple AMO to denitrification (Ettwig et al., 2008). Ettwig and colleagues (2009) reported another successful enrichment of denitrifying anaerobic

methane oxidation culture, dominated by the same bacteria. This culture was inoculated with sediments from ditches draining agricultural land. In the latter two studies, the cultures were fed with methane, nitrate and nitrite, as in Raghoebarsing and colleagues (2006), but operated at 30°C instead of 25°C (Ettwig et al., 2008; Ettwig et al., 2009). By metagenomic sequencing of the latter two cultures, Ettwig and colleagues (2010) assembled the complete genome of the dominant bacterial species, named '*Candidatus Methylomirabilis oxyfera*' (hereafter call '*M. oxyfera*').

Hu and colleagues (2009) reported successful enrichment of two denitrifying anaerobic methane oxidation cultures from a mixture of activated sludge and digester sludge, both taken from a wastewater treatment plant, and sediments from a freshwater lake. Both cultures were fed with nitrate as the electron acceptor and methane as the sole electron donor. Hu and colleagues (2009) showed that both cultures had similar nitrate reduction rates and nitrite reduction rates. This observation led the authors to hypothesize that these cultures may play an important role in nitrate reduction. Several literatures regarding denitrifying AMO microorganisms provide engineering potential for economic denitrification using methane gas that can be captured from organic waste and wastewater (e.g., anaerobic digestion). However, there are no studies on kinetic parameters of AMO-denitrifying microorganisms.

1.2 Objectives

This project aimed to:

- Enrich and develop the anaerobic methane oxidation (AMO) denitrifying cultures
- Demonstrate the denitrifying anaerobic culture for AMO will have a direct impact on the available nitrate (electron acceptor)
- Experimentally estimate kinetic parameters for AMO coupled to nitrate, since current available values do not exist and do not provide a clear view of whether AMO coupled to nitrate can be chosen as competitive process in biological denitrification in wastewater treatment.

1.3 Scopes

This project investigated the impacts of AMO denitrifying bacteria in a bench-scale set up results were used to develop a kinetic model. The scope of this project included:

- Operation of bench-scale reactors initially seeded with return activated sludge from the Alisha Craig WWTP and synthetic medium solution, and fed daily with CH_4 gas to incubate AMO denitrifying bacteria.
- Operation of several bench-scale pressure tubes fed with AMO denitrifying bacteria and CH_4 gas to establish total mass balance.
- Estimation of kinetic parameters for AMO coupled to nitrate in batch-scale reactors.

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2 Literature Review

2.1 Introduction

Nitrate contamination of water sources is becoming a problem in Canada as well as in other areas of the world (Buchheister, 2000). In many areas the nitrate concentration in water resources has reached serious levels exceeding the nominal limits of 10.0 mg/L as NO_3-N (nitrate nitrogen) set by the U.S. Environmental Protection Agency (Sayre, 1988) or 50 mg/L as NO_3^- (nitrate) set by the World Health Organization (WHO). Gillham (1992) indicated that urban sewage effluents can contribute up to 40% of the nitrates present in surface water. Moreover, nitrate levels have been increasing because of increased usage of fertilizers, changes in land-use patterns, and increased recycling of domestic wastewater.

Concern over the nitrate concentrations in water sources is increasing due to potential ill effects on human's health. Concentrations greater than 10 mg NO_3-N/L can be fatal to infants under six months of age. In infants, nitrate is reduced to nitrite, which combines with hemoglobin in the blood to form methemoglobin, and leads to a condition commonly known as "blue baby syndrome" (Walker, 1990). Increased nitrate concentrations in ground water have caused the shutdown of wells and have rendered aquifers unusable as water sources. Communities with closed nitrate-contaminated wells now need them to meet the increased water demand. Surface waters also have experienced seasonal nitrate violations. As a result, there is renewed interest in the removal of nitrates from raw water (Bouchard & Surampalli, 1992).

Nitrate is a stable and highly soluble ion with low potential for precipitation or adsorption. These properties make it difficult to remove nitrate using conventional water treatment technologies such as lime softening and filtration (Bouchard & Surampalli, 1992). Alternative technologies, for example, chemical denitrification, ion exchange, reverse osmosis, electrodialysis, catalytic denitrification, and biological denitrification can be used to remove nitrates from water sources (Jae & Young, 2009).The most promising and versatile approach for full-scale application being studied is biological denitrification (De Lucas, 2005), mainly due to its economical merits over other chemical/physical methods.

2.2 Biological denitrification

In biological nitrogen removal systems, ammonium nitrogen is oxidized to nitrite (NO_2^-) or nitrate (NO_3^-) by nitrifying bacteria in a process called nitrification. The oxidized nitrogen species are reduced to nitrogen gas by denitrifying bacteria in a process called denitrification. Many facultative, obligate anaerobic bacteria are capable of reducing oxidized forms of nitrogen species to nitrogen gas for energy generation (Gillham, 1991).

The enzymes associated with denitrification are synthesized when conditions become advantageous for denitrification. Synthesis of denitrifying enzymes is typically a highly regulated process. It is generally understood that denitrifying enzymes are inducible. Their synthesis occurs under anaerobic conditions, although denitrification can

occur in the presence of oxygen. Reduction of nitrate to nitrogen gas proceeds in four steps, according to the following scheme (Zumft, 1997):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Each step is catalyzed by an enzyme system. The reduction of nitrate to nitrite is important to a number of bacteria (e.g., *Thiobacillus denitrificans*, *Micrococcus denitrificans*, *Paracoccus denitrificans* and *Pseudomonas*) since the process involves energy conservation through generation of a proton motive force or by increased substrate-level phosphorylation reaction. This step is catalyzed by membrane bound nitrate reductases. The dissimilatory reduction of nitrite is carried out by two distinct nitrite reductases. Nitric oxide is typically produced from nitrite, but under some conditions (e.g., oxygen-poor soils and marine environments) nitrous oxide is also produced. Nitrite reductases are membrane-bound as well as cytoplasmic enzymes. The reduction of nitric oxide is the least productive enzymatic step associated with denitrification. But there are reports showing that nitric oxide reductase is present in bacterial membranes (Matějů, 1992). Literatures showed that nitrite reduction to nitrous oxide proceeds via nitric oxide and are catalyzed by two discrete enzymes, a nitrite reductase and a nitric oxide reductase (Gillham, 1991). The last denitrification step, the reduction of nitrous oxide to dinitrogen, is catalysed by nitrous oxide reductase. This step is coupled to ATP formation (Zumft, 1997). While considerable progress has been made in characterizing denitrification enzymes and much is known about the mechanism of denitrification, several areas still remain to be clarified. For instance, the mechanism by which the N-N bond of nitrous oxide is formed is unclear, even though the reactions have been studied intensively (Tavares, 2006).

From detailed studies of the enzyme systems of some bacterial species it can be concluded that the promoters that affect induction and repression of these enzymes are not universal because denitrifying bacteria are genetically diverse and metabolically versatile. There are still unknown phenomena on regulatory interdependence of reductases involved in each step of denitrification.

Since denitrification is a respiratory process, an electron donor is needed as an energy source. Denitrifying bacteria are mostly heterotrophs and utilize a variety of organic compounds as the electron donor, which include glucose, ethanol, acetate and so on. Some of them are capable of utilizing 1-carbon compounds (e.g., methanol). Although many of denitrifying bacteria are heterotrophs, there are some autotrophic denitrifying bacteria that utilize hydrogen gas, reduced iron, or reduced sulfur compounds (Rittmann & Huck , 1989; Lampe, 1999; Tavares, 2006).

2.2.1 Heterotrophic denitrification

Heterotrophic denitrifying bacteria require an organic carbon source for respiration and growth. A wide variety of organic compounds has been used, such as methanol, ethanol, glucose, acetate, aspartate, or formic acid (Koren, 2000), which act as electron donor. The stoichiometric relationships of various organic carbon substrates required for heterotrophic denitrification is listed in Table 2.1.

Table 2.1 Stoichiometric relationships of heterotrophic denitrification with various carbon substrates.

Substrate	Reaction
Ethanol[a]	$0.69\ C_2H_5OH + NO_3^- + H^+ \rightarrow 0.14\ C_5H_7NO_2 + 0.43\ N_2 + 0.67\ CO_2 + 2.07\ H_2O$
Methanol[b]	$0.1667\ CH_3OH + 0.1561\ NO_3^- + 0.1561\ H^+ \rightarrow 0.00954\ C_5H_7O_2N + 0.0733\ N_2 + 0.3781\ H_2O + 0.119\ CO_2$
Acetic acid[c]	$0.82\ CH_3COOH + NO_3^- \rightarrow 0.07\ C_5H_7NO_2 + HCO_3^- + 0.30\ CO_2 + 0.90\ H_2O + 0.47\ N_2$
Glucose[c]	$0.36\ C_6H_{12}O_6 + NO_3^- + 0.18\ NH_4^+ + 0.82\ H^+ \rightarrow 0.18\ C_5H_7NO_2 + 0.5\ N_2 + 1.25\ CO_2 + 2.28\ H_2O$
Propanol[d]	$0.278\ C_3HOH + NO_3 \rightarrow 0.5\ N_2 + 0.833\ CO_2 + 0.611\ H_2O + N_2$
"Typical" organic matter[c]	$0.3\ C_5H_3NO + NO_3^- + H^+ \rightarrow 0.11\ C_5H_7NO_2 + 0.5\ N_2 + 0.95\ CO_2 + 1.17\ H_2O + 0.19\ NH_4^+$

[a] McAdam and Judd 2006; [b] Buchheister 2000; [c] Matějů et al. 1992; [d] Dillon et al. 1991

Heterotrophic denitrification has been widely used for controlling nitrogen in wastewater treatment (De Lucas, 2005), typically called biological nitrogen removal (BNR) systems. A nitrification step is essential before denitrification for BNR systems, since nitrogen compounds in wastewaters mainly exist as organic nitrogen or ammonium nitrogen (Jae & Young, 2009). Nitrification is the process by which ammonia nitrogen is converted to nitrites (NO_2^-) and then nitrates (NO_3^-). This process naturally occurs in the environment, is carried out by nitrifying bacteria. The equations of nitrification are shown below:

$$2\,NH_3 + 3\,O_2 \rightarrow 2\,NO_2^- + 2H_2O + 2\,e \quad (1.1)$$

$$2\,NO_2^- + O_2 \rightarrow 2\,NO_3^- \quad (1.2)$$

Nitrification requires oxygen for oxidizing reduced nitrogen species into nitrate, which is a main operating and capital cost in biological nitrogen removal systems.

Pre-denitrification has normally employed in Biological Nutrient Removal (BNR) systems, which recycles nitrate from aeration tank back to anoxic tank (see Figure 2.1), because organic compounds in wastewater are used for electron donor in denitrification. Post-denitrification that locates denitrification tank after aeration tank can be used in some cases where effluent nitrogen standard is very strict (< 3 mg/L). Post-denitrification needs an exogenous electron donor due to the lack of organics after aerobic oxidation, and the costs for the electron donor can be substantial.

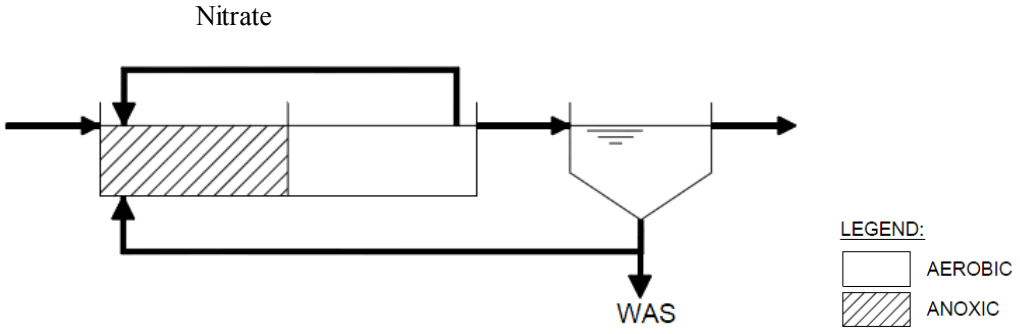


Figure 2.1 Pre-denitrification process.

The addition of exogenous electron donor to denitrification is essential for some industrial wastewater processes (e.g., mineral processing, electroplating, semiconductor manufacturing, and power plants) because these industrial wastewaters typically contain very low concentrations of carbon compounds (~130 g DOC/L), often no carbon at all, together with high concentration of nitrate (1.4 - 40.0 g NO_3/L) (Buchheister, 2000). For such industrial wastewaters, post-denitrification is the only way for nitrogen control. As a result, the expense for exogenous electron donor can be more substantial.

In addition, the remaining exogenous electron donor in denitrification can increase the concentration of biochemical oxygen demand (BOD) in final effluents from BNR systems, this causes secondary contamination which requires supplemental post-treatment processes would be required to meet effluent standards (Matějů, 1992).

One option for decreasing the cost for exogenous electron donor is to use cheaper organics, such as methanol, cotton, wheat straw, newspaper, sawdust, starch, and vegetable oil (Annalisa & April. 2008). Unfortunately, these cheap organics have a low solubility that can limit denitrification rates; the investment for large footprint will trade off the reduction of maintenance costs for exogenous electron donor (Soares, 2000). The other option is to use electron donors that can be recovered from wastewater streams, such as methane; anaerobic digestion has been widely used for high strength organic wastes and wastewaters. Methane is not expensive, and available from wastewater treatment plants (e.g., sludge digester) or landfill leachate. In the latter two cases (wastewater and landfill leachate) methane would be especially suitable, since it is generated onsite due to the anaerobic digestion of sludge in wastewater treatment plants and anaerobic degradation of organic waste in landfills (Oskar Modina, 2007).

2.2.2 Autotrophic denitrification

Paracoccus, Thiobacillus, and Thiosphaera can accomplish denitrification autotrophically using hydrogen or various reduced sulfur compounds (e.g., S°, S^{2-},$S_2O_3^{2-}$, $S_4O_2^{2-}$ $S_20_3^{2-}$, $S_40_2^{2-}$, or SO_3^{2-}) as electron donors (De Lucas, 2005), while they use inorganic carbon (CO_2 or HCO_3^-) as carbon source. Bacteria from the genera *Ferrobacillus, Gallionella, Leptothrix*, and *Sphaerotillus* can utilize ferrous iron as an energy source for autotrophic denitrification. Stoichiometric equations of autotrophic denitrification with various energy sources are listed in Table 2.2.

Table 2.2 Stoichiometric relationships of autotrophic denitrification with various electron donors.

Electron donor	Reaction
Hydrogen[a]	$3.03\ H_2 + NO_3^- + H^+ + 0.23\ CO_2 \rightarrow 0.05\ C_5H_7NO_2 + 0.48\ N_2 + 3.37\ H_2O$
Sulfur[b]	$1.10\ S + NO_3^- + 0.76\ H_2O + 0.4\ CO_2 + 0.086\ NH_4^+ \rightarrow 0.04\ C_5H_7NO_2 + 0.48\ N_2 + 0.98\ SO_4^{2-} + 0.96\ H^+$
Thiosulfate[b]	$0.84\ S_2O_3^{2-} + NO_3^- + 0.43\ H_2O + 0.35\ CO_2 + 0.87\ HCO_3^- + 0.087NH_4^+ \rightarrow 0.087\ C_5H_7NO_2 + 0.5\ N_2 + 1.69\ SO_4^{2-} + 0.7\ H^+$
Hydrogen sulfide[b]	$0.421\ H_2S + 0.421\ HS^- + NO_3^- + 0.346\ CO_2 + 0.086\ HCO_3^- + 0.086\ NH_4^+ \rightarrow 0.842\ SO_4^{2-} + 0.500\ N_2 + 0.086\ C_5H_7NO_2 + 0.434\ H_2O + 0.262\ H^+$
Ferrous ion[c]	$NO_3^- + 5\ Fe_2+ \rightarrow 0.5\ N_2 + 5\ Fe(OH)_2 + 9\ H^+$

[a] McAdam and Judd 2006; [b] Hashimoto et al. 1987; [c] Ghafari et al. 2008

Under autotrophic growth conditions, carbon dioxide or bicarbonate is used as carbon source for bacteria growth (Lampe, 1999). *Thiobacillus* denitrificans, representative to autotrophic denitrifiers using reduced forms of sulfur compounds, were used to reduce nitrate concentrations from 24 to 1 mg NO_3^-/L in packed bed reactors using elemental sulfur as electron donor. Lewandowski (1987) encapsulated autotrophic denitrifiers in calcium alginate beads containing sulfur and calcium carbonate for autotrophic denitrification, and he reported that nitrate concentrations were reduced from 27 to 6 mg/L in seven hours. Lee and Rittmann (2000) described a novel hollow-fiber membrane-biofilm reactor (HFMBR) that is especially well suited for autotrophic denitrification using H_2. Hydrogen gas is supplied to the inside of hollow-fiber membranes that have a hydrophobic and non-porous inner layer. H_2 diffuses through the wall of the membrane and dissolves into the aqueous phase on the outside of the membrane. When electron acceptors, such as nitrate, are present in the liquid, a biofilm of H_2-oxidizing autotrophs forms naturally on the outside of the membrane. Two key advantages for carrying out autotrophic denitrification stem from the counter-current diffusion of H_2 and nitrate that occur naturally with the HFMBR. First, nearly 100% utilization of H_2 is possible. This minimizes the cost of supplying the electron donor and the residual concentration of H_2 in the effluent. It also prevents formation of H_2 bubbles and an explosive atmosphere in the reactor. Second, counter-current diffusion allows high fluxes of nitrate and H_2, which minimize the retention time for the reaction needed.

2.3 AMO coupled to nitrite reduction

Anaerobic methane oxidation (AMO) is a microbial process that occurs mainly in anoxic marine sediments. It is considered to be a very important process that can reduce the emission of methane from the ocean into the atmosphere. It is estimated that almost 90% of all the methane that arises from marine sediments is oxidized anaerobically by this process (Reeburgh, 2007). Most common way of AMO is that methane is oxidized with sulfate as the terminal electron acceptor:

$$CH_4 + SO_4^{2} \rightarrow HCO_3^{-} + HS^{-} + H_2O \quad (2.3)$$

The process of sulfate-mediated AMO is performed by microbial consortia between methanotrophic archaea and sulfate-reducing bacteria via the syntrophic transfer of elemental sulfur (Milucka, 2012). The research on AMO seems to be limited because those AMO microorganisms have not been isolated; they are extremely slow growers having a minimum doubling time of about a few months. Thus, many aspects of the physiological mechanism of AMO remain unknown (Luesken, 2011).

Although sulfate is still considered as the most important electron acceptor for AMO, there are other alternative electron acceptors for AMO microorganisms. CH_4 oxidation coupled to the reduction of manganese, iron, nitrate, or nitrite yields at least 10 times more energy than sulfate reduction (Ettwig v. P.-S., 2009). AMO coupled to denitrification has been documented in polluted canals and lake sediments, though the mechanism of this process is still unclear (Milucka, 2012). The lack of experimental evidences for the occurrence of AMO coupled to denitrification is not surprising because this process is expected to occur close to the oxic/anoxic interface in sediments (Kampman, 2012). This interface is generally characterized by steep gradients, occurring

within millimeters, masking the process from geochemical detection. Furthermore, laboratory enrichment of the responsible microorganisms could be difficult because of their extremely slow growth rate. Recent findings suggest that denitrification coupled to methane oxidation by a single bacterial species does not require the archaea partner (Ettwig et al. 2008). The bacteria involved in the nitrate reducing process are not directly related to the AMO microorganisms found in marine sediments where methane oxidation is coupled to sulfate reduction. The AMO coupled to denitrification is shown in equations 2.4 and 2.5 (Knowles, 2005).

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \ \left(\Delta G^{0'} = -765\, kJ\, mol^{-1} CH_4\right) \quad (2.4)$$

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \ \left(\Delta G^{0'} = -928\, kJ\, mol^{-1} CH_4\right) \quad (2.5)$$

In 2006, Raghoebarsing and colleagues first reported a nitrite dependent anaerobic methane oxidization (NDAMO) enrichment culture from anoxic fresh water sediment (Raghoebarsing et al., 2006). In this culture, one bacterial phylotype belonging to the candidate division "NC10" constituted 80% of the population. This division had been formed only by environmental sequences (Rappé and Giovannoni, 2003). A smaller fraction of the population (up to10%) consisted of archaea that were distantly related to the AMO archaea of group2. The experiments suggested that both the bacteria and the Archaea were involved in the NDAMO reaction (Raghoebarsing et al., 2006). However, later studies revealed that the NDAMO reaction could be performed by the single bacterial species (Ettwig et al., 2008, 2009). Nitrite was found to be a key component in selecting bacterial species (Hu, 2009). In 2010, Ettwig and colleagues assembled the

complete genome of the bacterial species responsible for the NDAMO process, named "*Candidatus Methylomirabilis oxyfera*" (Ettwig et al., 2010).

Several enrichment cultures of *M. oxyfera* have been obtained from different fresh water habitats (Table1). *M. oxyfera* cells possess a cell envelope typical of Gram-negative bacteria with a diameter of 0.25–0.5 µm and a length of 0.8–1.1 µm (Ettwig K. P.-S., 2009). The measured apparent affinity constant for methane of *M. oxyfera* is smaller than 5 µM (Ettwig et al., 2008) or even smaller than 0.6 µM (Raghoebarsing et al.,2006), which is significantly lower than the affinity of sulfate dependent AMO for methane (in the order of mM; Nauhaus e tal.,2002). However, the affinity of sulfate dependent AMO for methane described by Nauhaus et al. (2002) were quantified by the marine sediments, which were not continuously shaken. This may cause diffusion limitations compared to the well mixed systems (Ettwig et al., 2008; Raghoebarsing et al., 2006). The specific activity of *M. oxyfera* is low, 0.9–6.2 nmol NO_2^- min^{-1} mg $protein^{-1}$ (Table1.3). In addition, the observed growth rate of *M. oxyfera* is low, with a doubling time of 1–2 weeks (Ettwig et al., 2009).

Table 2.3 the reported enrichment cultures of *M. oxyfera*.

Inoculum	Temperature (◦C)	Convention rate (nmol NO_2^- min^{-1} mg $protein^{-1}$)	Composition (%)		Reference
			Bacteria	Archaea	
Canal sediments	25	6.2	80	10	(Raghoebarsing, 2006)
Canal sediments	30	3.7	70	0	(Ettwig K. P.-S., 2008)
Ditch sediments	30±1	Not reported	70-80	-	(Kampman, 2012)
Ditch sediments	30	3.4-5.6	70	0	(Ettwig K. P.-S., 2009)
Mixed inoculum	35	Not reported	15	0	(Hu, 2009)
Wastewater sludge	20-23	0.9	60-70	-	(Luesken, 2011)

The NDAMO process is one of the latest findings linking the carbon and nitrogen cycles. The detailed physiological and biochemical properties of this bacterium remain unclear because of the limited availability of enrichment cultures. The acquisition of a greater number of enrichment cultures from various habitats and pure cultures of *M. oxyfera* would be helpful in unraveling the unexplored parts of this bacterium. Furthermore, the existence of the intra-aerobic pathway needs to be further examined by isolation and identification of the key enzyme(s) responsible for the conversion of NO to N_2 and O_2 (Ettwig et al. 2009).

The NDAMO process can potentially have significant economic and environmental benefits if is combined with anaerobic wastewater treatment producing methane; the methane generated from wastewater can be used for exogenous electron acceptor for denitrification. This new concept can save operating and maintenance costs significantly in one hand. In the other hand, nitrogen is successfully controlled in wastewater treatment. There are, however, several challenges to be explored for engineering NDAMO. For instance, the slow growth rate of *M. oxyfera* need substantial footprint for denitrification step, which increase capital costs over conventional denitrification processes. In addition, the time required for enriching NDAMO culture takes 8–16 months (Raghoebarsing et al., 2006; Ettwig et al., 2009; Hu, 2009), which indicates that the start-up of engineered NDAMO systems is unrealistic. Finally, NDAMO microorganisms prefer nitrite to nitrate as electron acceptor, which indicates that partial nitrification (from ammonium to nitrite) or new microorganisms would be required.

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2 Materials and Methods

3.1 Development of a microbial community that couples the AMO to denitrification in batch reactors

Sediment samples from return activated sludge（RAS）were obtained from Alisha Craig Wastewater treatment plant as inoculum. The sediment was incubated in three bottles (1L of working volume) (Figure 3.1). Initially, 50 ml of sludge and 450 mL of synthetic medium solution were added into each bottle. Different carbon sources were used in the three bottles: methane for bottle #1, mixture of methane and acetate (1 mmol/L) for bottle #2, and no carbon source for bottle #3. Bottle #3 was purged with 99% helium gas, the other bottles were purged with methane gas. Biogas produced from bottles was collected with a gas bag. The bottles #1 and #2 were purged with methane gas (99%) daily (for 20 min at a flow rate of 500mL/min), and the bottle #3 was purged with helium gas (99%). Gas was recirculated using a peristaltic pump to provide mixing and sufficient gas transfer. The three bottles were incubated in the dark at 37 °C on a shaker (SI-300, Lab Companion, USA) at 150 rpm.

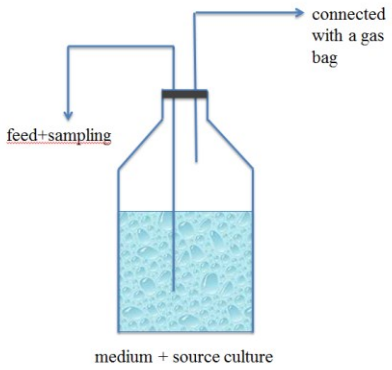


Figure 3.1 schematic of fed-batch reactor.

Table 3.1 showed the compositions of the medium solution. The trace mineral solution contained, per liter: 100 mg $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $NiCl_2 \cdot 6H_2O$, and 30 Na_2SeO_3. The prepared medium was sterilized in the autoclave, and then the pH was adjusted at 7.4 ± 0.2 using 1 N H_2SO_4. The medium solution was then flushed with 99% CH_4 gas for 20 minutes to reach saturation state.

Table 3.1 Compositions of the medium.

Chemical	Concentration (g/L)
KH_2PO4	0.128
Na_2HPO_4	0.434
$NaNO_3$	0.685
$Na_2S \cdot 9H_2O$	0.4804
$CaCl_2 \cdot 2H_2O$	0.001
$FeSO_4 \cdot 7H_2O$	0.001
$MgCl_2 \cdot 6H_2O$	0.1647
Trace solution	1 mL

Liquid samples (10 mL) were collected from each bottle every 24 hours before purging the methane for water quality analysis.

To enrich more anaerobic denitrifying methanotrophic microorganisms , 200 mL supernatant from both of bottles #1 and #2 were collected and transferred to other serum bottles (500 mL working volume). These bottles were filled with new medium and methane gas was provided in the same manner as previous described experiments. This

enrichment was repeated three times more to concentrate the anaerobic denitrifying methanotrophic microorganisms.

3.2 Mass balance tests

To improve data accuracy for mass balance experiments the denitrifying microorganisms enriched in serum bottles (500 mL working volume) were transferred to small vials (25 mL working volume). Supernatant collected from serum bottles were centrifuged with 1.5 mL micro-centrifuge tubes at 12,000 rpm for 10 minutes to form pellets. The pellets were washed with medium solutions and 0.1 M KH_2PO_4 solution several times, and re-suspended with DI water in the micro-centrifuge tubes. Then, the pellets and medium solution were transferred to small vials, and methane gas was provided for the vials in 20 min. Then, the vials were incubated in the shaker at 37 °C and 150 rpm for 120 hours.

After 5 days' incubation, 0.5 mL gas sample were collected from headspace to measure the composition of N_2, CH_4, CO_2, N_2O. Liquid sample of 10 mL was collected to measure pH, nitrate and nitrite concentration. Remaining liquid sample of 4 mL was taken out and transferred to new vials for repeating mass balance tests. These experiments were conducted in quintuplicate.

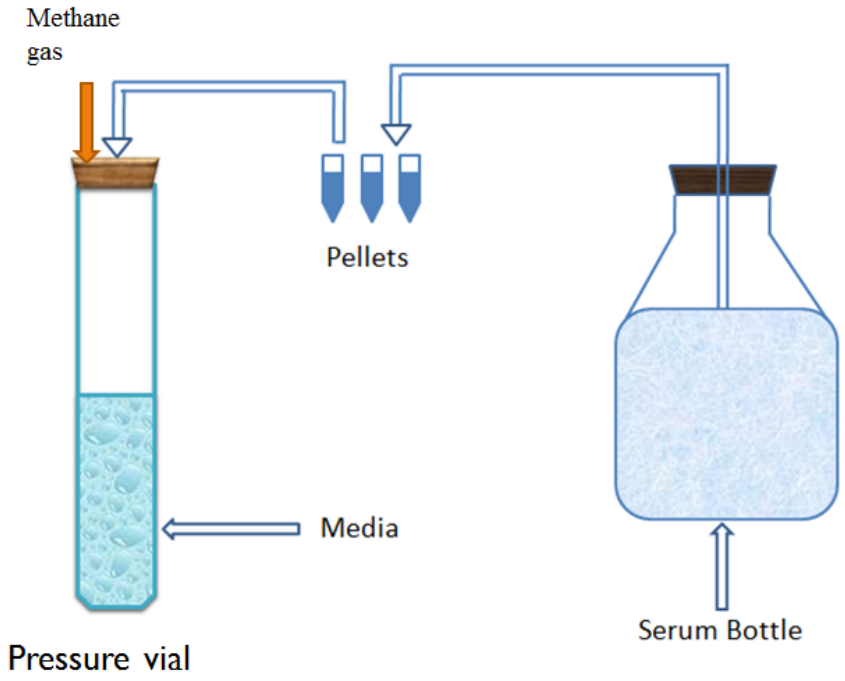


Figure 3.2 Schematic of mass balance test.

3.3 Kinetics test

Experiments were conducted to estimate kinetics parameters that include the maximum specific growth rate (μ_{max}), the maximum specific rate of substrate (q_{max}), the half-maximum-rate concentration (K_s), and the endogenous-decay coefficient (b). With μ_{max} and q_{max} values biomass growth yield (Y) was calculated.

Equations 3.1 and 3.2 show how these parameters describe the growth of active biomass (X_a in mg-cells/L) and consumption of CH_4 (S in mg COD/L):

$$\frac{dX_a}{dt} = \left[\mu_{max} * \left(\frac{S}{K_s+S}\right) - b\right] * X_a = \left[Y * q_{max} * \left(\frac{S}{K_s+S}\right) - b\right] * X_a \quad (3.1)$$

$$\frac{\mathrm{dS}}{\mathrm{dt}} = -q_{max} * \left(\frac{S}{K_S + S}\right) * X_a \quad (3.2)$$

3.3.1 Estimation of μ_{max} (the maximum growth rate)

The maximum growth rate (μ_{max}) was determined using equation 3.3 (a simplified form of equation 1 for S»Ks because the term S/(Ks+S) becomes 1.

$$ln\left(\frac{Xt}{X}\right) = \mu_{max} * t \quad (3.3)$$

Where X is the initial biomass concentration, X_t is the biomass concentration at time (t) during the exponential growth with non-limiting CH_4 concentrations.

This experiment was conducted in duplicate; two identical 1L media bottles (500 mL working volume) were used for the tests (see Figure 3.1). 50 mL of culture and 450 mL of medium solution (see Table 3.1) were added into each bottle. Each bottle was purged with 99% CH_4 gas for 20 min at the beginning of the experiments. The two bottles were then placed in the shaker at 37°C and 150 rpm for 8 days. To keep non-limiting substrate (methane) conditions the bottles were purged with methane for 20 min once every day after collecting gas and liquid samples. Gas samples (0.5 mL) were collected every 24 hours to measure CH_4, N_2 and CO_2 gas in the headspace. Liquid samples (10 mL) were collected to measure biomass, dissolved-oxygen, nitrate, nitrite, dissolved-CH_4 concentrations and pH.

3.3.2 Estimating q_{max} (the maximum specific substrate utilization rate)

The maximum specific substrate utilization rate (q_{max}) was calculated using equation 3.4:

$$q_{max} = -\left(\frac{1}{Xa*V_L}\right)\left(\frac{d[CH4]_L}{d_t}\right)\left(\frac{V_G}{R*T*K_H} + V_L\right) \qquad (3.4)$$

Where V_L is the liquid volume (L), V_G is the headspace volume (L), R is the universal gas constant (0.082 L-atm/mol-K), and T is temperature in Kelvin, and K_H is the Henry's Law constant in mol/L-atm.

To obtain a reliable q_{max} value directly, the CH_4 concentration in the system must be significantly greater than the Ks value so that the (S/(Ks+S)) term can be simplified to 1. In addition, net biomass growth should be small for the duration of the experiment so that biomass term, Xa, can be set to as a constant value. To meet these criteria, the rate of CH_4 utilization in a batch was non growth experiment.

q_{max} experiments were conducted with 250 mL of fresh medium and inoculated with 50 ml of the source culture in four 500 mL serum bottles. Hence, the bottles had 300 mL of liquid volume and 200 mL of gas phase. The bottles were purged with CH_4 gas and kept on the shaker for two days to measure biomass growth.

The experiments for q_{max} quantification were performed after the biomass started to increase at the beginning of the experiment, bottles were pressurized with CH_4 and the CH_4 consumption and biomass were monitored over time.

3.3.3 Estimate b (the endogenous decay constant)

Endogenous decay determine the oxidation of biomass to support cell maintenance. To obtain the endogenous decay constant experimentally, experiments were conducted under non-limiting CH_4 conditions to reach exponential growth phase. At the beginning of experiments, the headspace of each tube was flushed with 99% CO_2 gas to remove all the CH_4 and N_2 gas. The headspace gas compositions were monitored and the experiment was considered "a starting point at t=0" once the N_2 and CH_4 concentrations were undetectable. Biomass, CH_4, and N_2 concentrations were measured every day for 8 days. Because there was no CH_4 present at the beginning of the experiment, the mass balance simplifies to:

$$\ln\left(\frac{X_0}{X_t}\right) = b * t \qquad (3.5)$$

Where Xo is the initial biomass concentration and X_t is the X_a value at time t of the decay experiment.

3.3.4 Estimate Ks (the half maximum-rate substrate concentration)

To estimate the K_s, batch growth experiments were carried out under CH_4 rate-limiting conditions. The two critical conditions for K_s experiments are initial CH_4 and biomass concentrations. If initial $[CH_4]_L > Ks$, $[CH_4]_L/(Ks + [CH_4]_L)$ equals 1; thus, finding a unique Ks value is not possible. However, if initial $[CH_4]_L \sim= Ks$, then the rate of the reaction could be considered as first order . The second important condition is initial biomass concentration, which should be large enough so that CH_4 utilization can be monitored in a given time (in a few days).

Three 500 mL bottles with 250 mL of fresh medium and 50 ml of the source culture were purged with 99% CH_4 for a very short time (10 seconds- 30 seconds) then purged with 99.99% helium gas. Three experiments having initial CH_4 concentrations of 130, 119and 90 μM were performed. Because μ_{max}, q_{max}, Y and b were already estimated from the preceding experiments, Ks was found by fitting the experimental biomass growth data into model simulations.

3.4 Analytical methods and calculations

Ammonium concentration was measured by an Auto Analyzer 3 (Bran-Luebbe, Germany) that quantifies ammonium nitrogen and dissolved ammonia. pH was measured with a Bench top pH Meter (Model 420A, Orion Research Inc., USA). Nitrate Concentration was measured by using HACH nitrate analysis kits (21061-69, 0.3 to 30.0 mg/L NO3-N, Hach Company, USA). Nitrite concentration was also measured with HACH nitrite analysis kits （21075-69，2 to 250 mg/L NO_2^-, HACH Company, USA）

Gas samples were collected from the headspace of tubes using a gastight syringe and were analyzed for CH_4 , N_2, CO_2 with gas chromatography (SRI 310C, SRI instruments, USA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (PorapakQ, 6 ft x 1/8 inches, 80/100 mesh, Agilent Tech., USA). The temperatures of the column and the TCD detector were 41 and 200˚C, respectively. Helium was used as carrier gas at a flow rate of 10 mL/L at the pressure of 21 psi. N_2O was also analyzed by gas chromatography GC 2014 (Shimadzu, Japan) equipped with an electron capture detector (ECD) and a molecular sieve column (6 ft x 1/5 inches, 85/100 mesh, All tech, USA). The temperatures of the column and the ECD detector were 80^o C and 250^o C, respectively.

The concentration of dissolved methane in sample was quantified according to the modified methodology suggested by Kampbell and Vandegrift (1998). 10mL of permeate was transferred to vials (20 mL) which were already sparged with helium gas (99.999%, PraxAir, Canada) for 20 min to keep anaerobic conditions. During sample transfer to the vials atmosphere's pressure was maintained in the vials by releasing vial pressure to a small water bottle (160 mL) connected with a needle. Then the vials were placed in an incubation shaker (VWR Incubating Orbital Shaker, VWR International Inc., Canada) for a day to allow equilibrium between headspace and liquid phase at 37◦C and 170 rpm. After equilibrium headspace gas was injected from the vials to the GC-TCD using a gastight syringe (Hamilton Gastight High-Performance Syringe, Hamilton) USA). The concentrations of dissolved methane in permeate were calculated by Equation 3.6：

$$CH_4(aq) = \left(C_{CH_4} \times P \times MW_{CH_4} \times \frac{1000\ mg}{1g}\right) + \left(C_{CH_4} \times V_{head} \times MW_{CH_4} \times \frac{1000\ mg}{1g} \times T_0\right) \times \frac{1}{V_{head} \times (\frac{22.4L}{1mol}) \times (\frac{1000mL}{1L}) \times T_1} \quad (3.6)$$

where $CH_{4(aq)}$ is the concentration of dissolved methane in AnMBR permeate (mg/L), C_{CH4} is the CH_4 percentage in headspace of vial, P is the pressure (1 atm), K_{CH4} is the Henry's law constant at 37◦C (0.0016 mol/L-atm), MW_{CH4} is the molecular weight of CH_4 (16 g/mol), V_{head} is the headspace volume of vial (10 mL), T_0 = 273.15 K, and T_1 = 310.15 K.

The biomass concentrations were determined from optical density (OD) measured with a spectrophotometer (Genesys 10s UV-vis, Thermo Scientific, USA) at wavelength of 600 nm. To establish a standard curve (Figure 3.3) between optical density reading and cell dry weight. Samples contain different biomass concentrations were centrifuged at 10,000 gpm for 10 minutes. Sedimented cells were washed three times in distilled water containing 15 mg $NaHCO_3$/L, then transferred to aluminum dishes, dry overnight in an oven at 100° C and weighed. The correlation between OD and cell dry weight was linear: (mg cells/L) = 1380.3* (OD) + 2.8723 (R^2=0.9916)

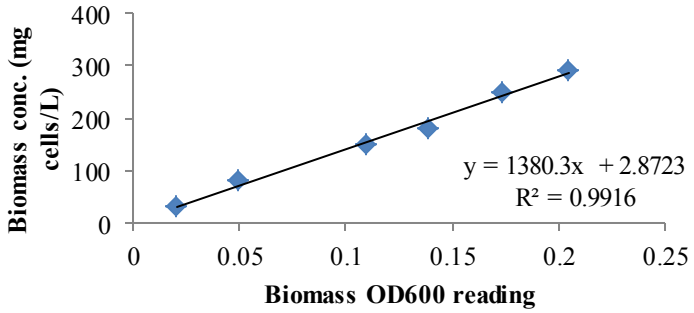


Figure 3.3 Relationship between OD reading and cell dry weight

4. Results and discussion

4.1 Development of a microbial community that couples the AMO to denitrification in batch reactors

Figure 4.1 showed that the nitrate concentration in bottle #1 (methane) and #2 (methane with acetate) declined from 3.1 mmol NO_3-N/L to 2.7 mmol NO_3-N/L and 1.8 mmol NO_3-N/L respectively, while nitrate concentration in bottle #3 (control) was decreased from 3.2 mmol NO_3-N/L to 3.1 mmol NO_3-N/L due to the oxidation of organic compounds from the inoculum or the mineral medium. The result of bottle # 1 indicates that the methanotrophs for anaerobic denitrification existed but the activity of the methanotrophs was relatively low, which indicated the biomass concentration of methanotrophs was low at the beginning of the test. The magnitude of decrease of nitrate concentration in bottle # 2 was significantly larger than bottle #1, which may suggest that acetate was also consumed as the electron donor for denitrification. The results also suggested that acetate may be more efficient compared to methane in the competition of methontrophs in anaerobic denitrification.

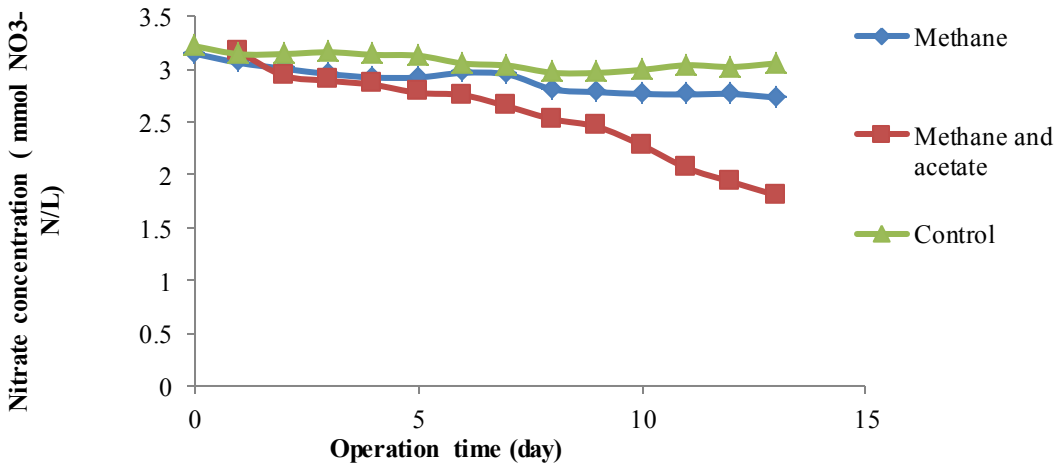
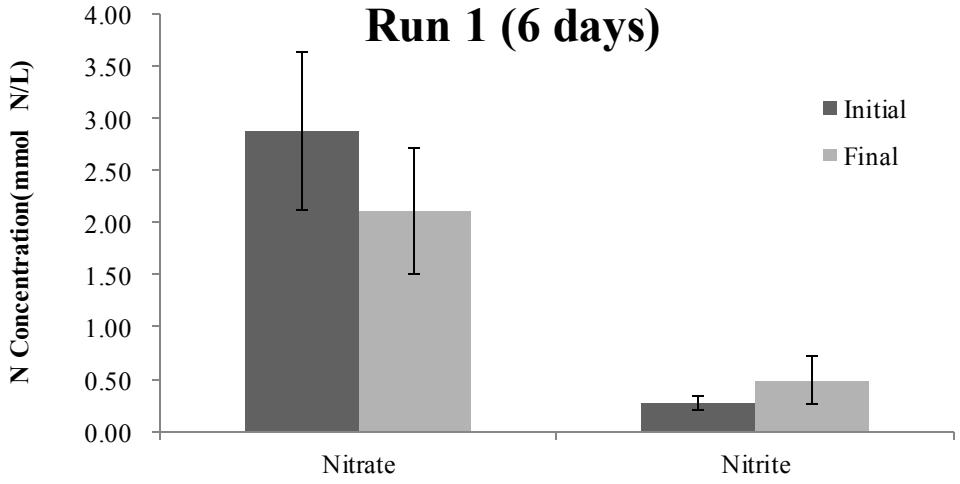


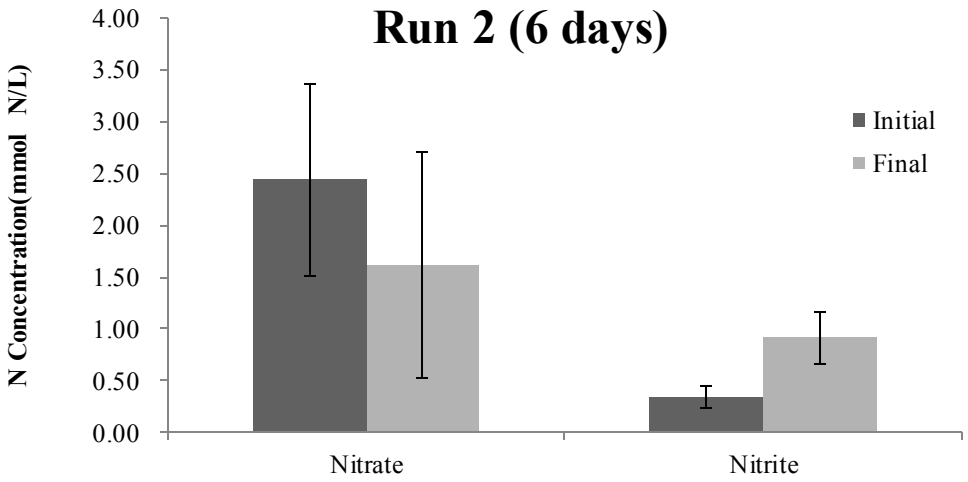
Figure 4.1 Nitrate concentrations in batch reactors.

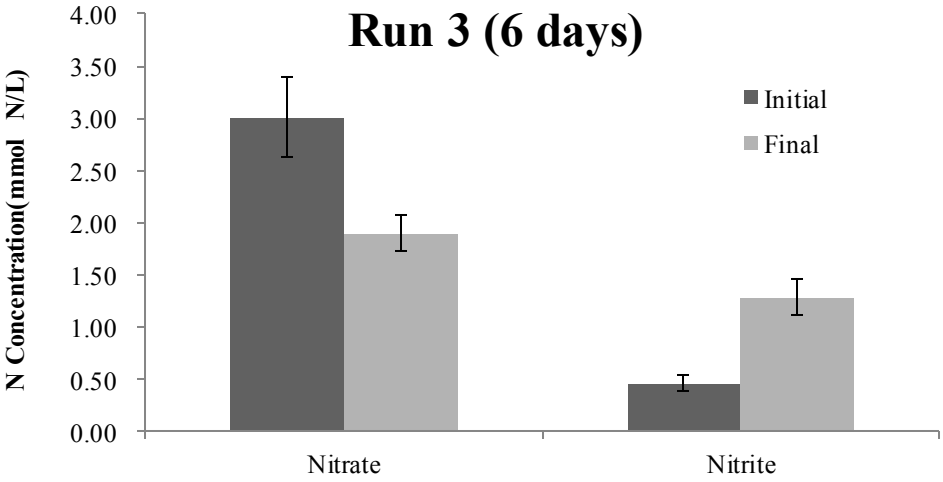
4.2 Mass balance tests

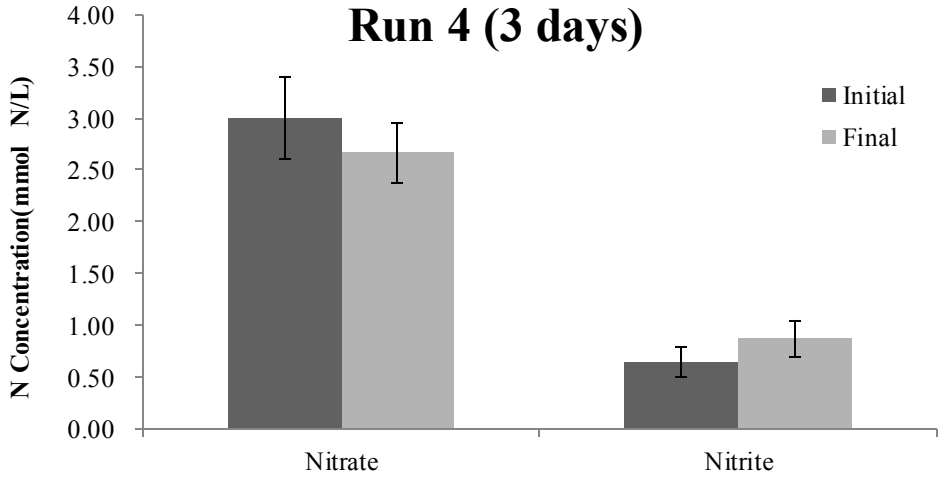
There were five runs in mass balance tests. The first three runs were operated for six days. The fourth run was for three days and the fifth run was for five days.

Figure 4.2 showed initial and final nitrate and nitrite concentrations in the mass balance test. The first three runs' data were similar; nitrate concentration was decreased from ~3 mmol NO_3-N/L to ~2 mmol NO_3-N/L in six days. It suggests that the methanotrophs' biomass was increased and as a result denitrification was enhanced after several runs of incubations compared to the first test. The increase of nitrite concentration in all of five runs indicates that part of nitrate was reduced to nitrite by the AMO coupled to denitrification.




Run 2 (6 days)
N Concentration(mmol N/L)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Initial
Final
Nitrate
Nitrite


Run 3 (6 days)
N Concentration(mmol N/L)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Initial
Final
Nitrate
Nitrite


Run 4 (3 days)
N Concentration(mmol N/L)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Initial
Final
Nitrate
Nitrite

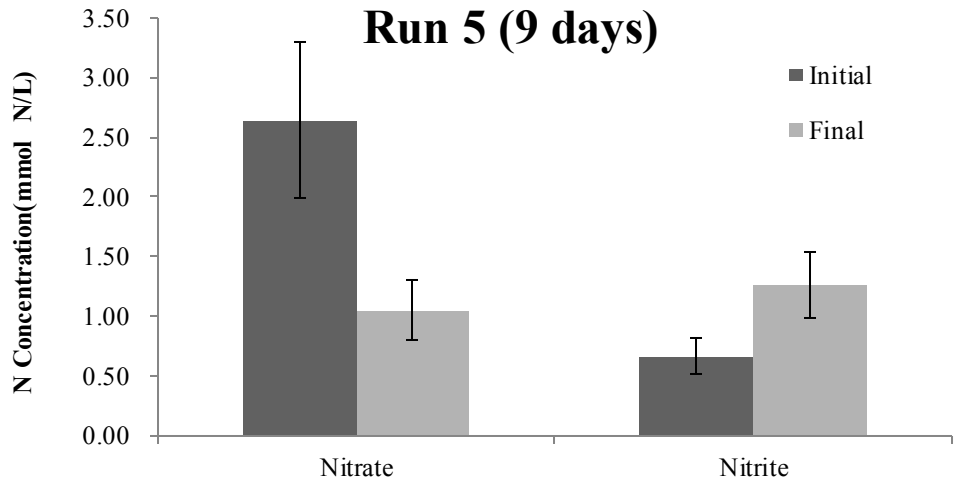
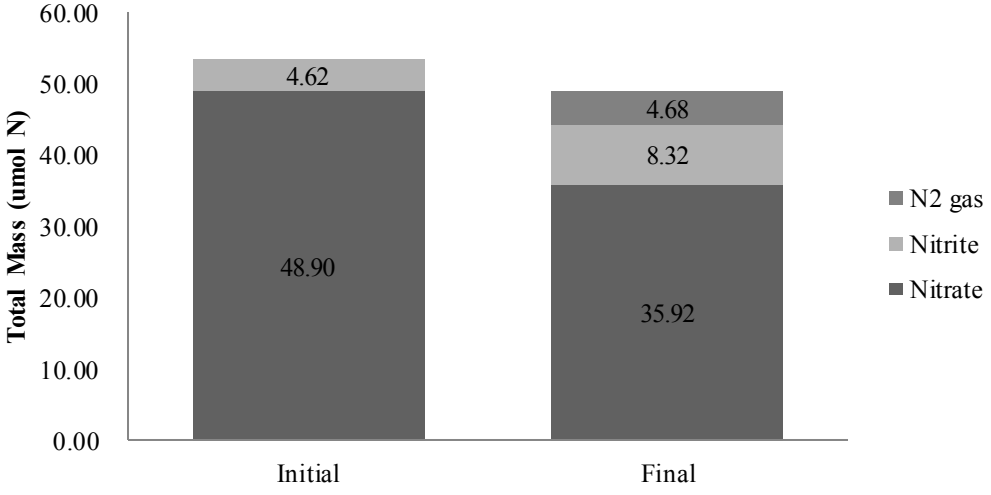


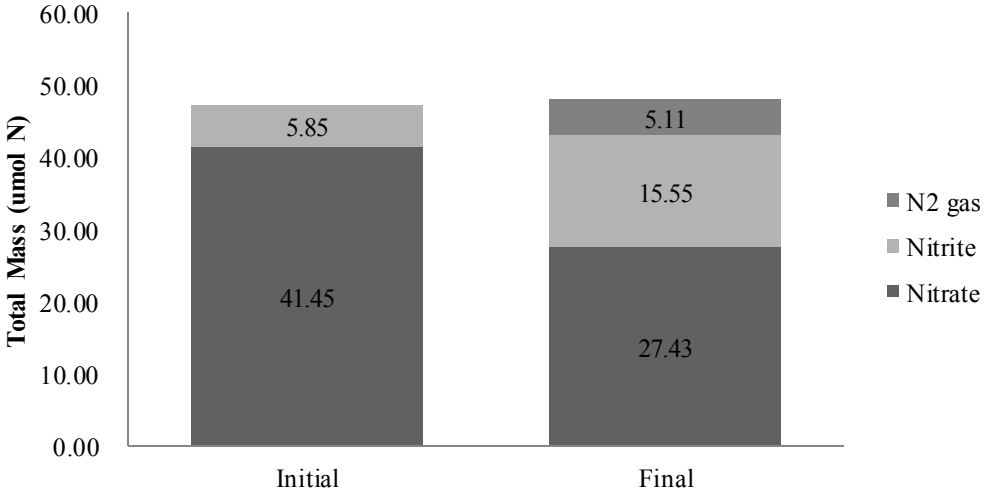
Figure 4.2 Nitrate and nitrite concentrations in mass balance tests.

Figure 4.3 showed the initial and final total nitrogen mass in five tests. All of the detected N_2 gas in the headspace of the culture was accounted for by the consumption of nitrite and nitrate since there is no oxygen in the tubes (no air intrusion). By using GC-ECD it was confirmed that there was no accumulation of N_2O gas in experiments. Figure 4.3 showed that in all five tests the final total nitrogen mass approximately equalized the total initial nitrogen mass. In addition, the stoichiometry of nitrate consumption coupled to denitrification was in a good agreement with equations 2.4 and 2.5. The percentage of nitirite in final total nitrogen mass increased with reaction time (3 days: 24% to 6 days: 30% to 9 days: 38%). The accumulation of nitrite indicates that the rate of nitrate reduced to nitrite is faster the rate of nitrite reduced to N_2 gas.

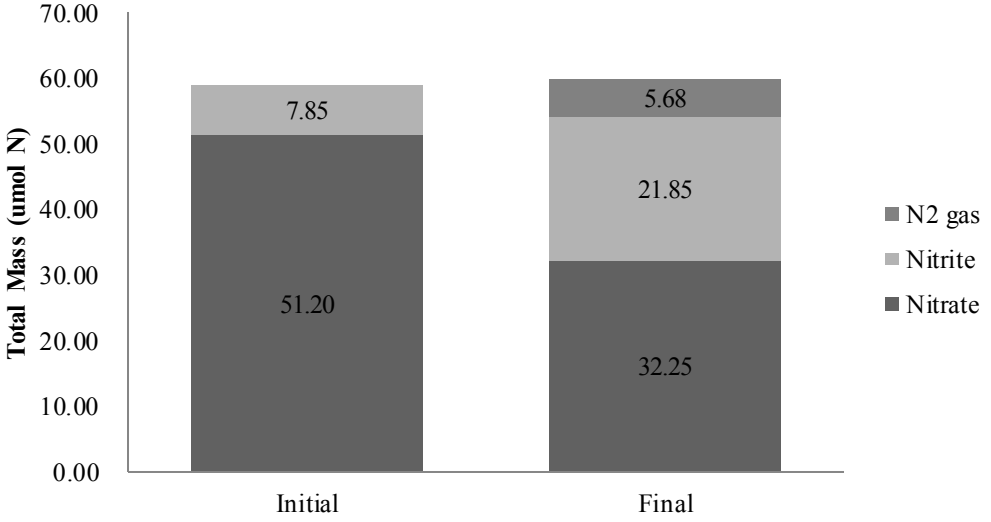
Run 1 （6 days）



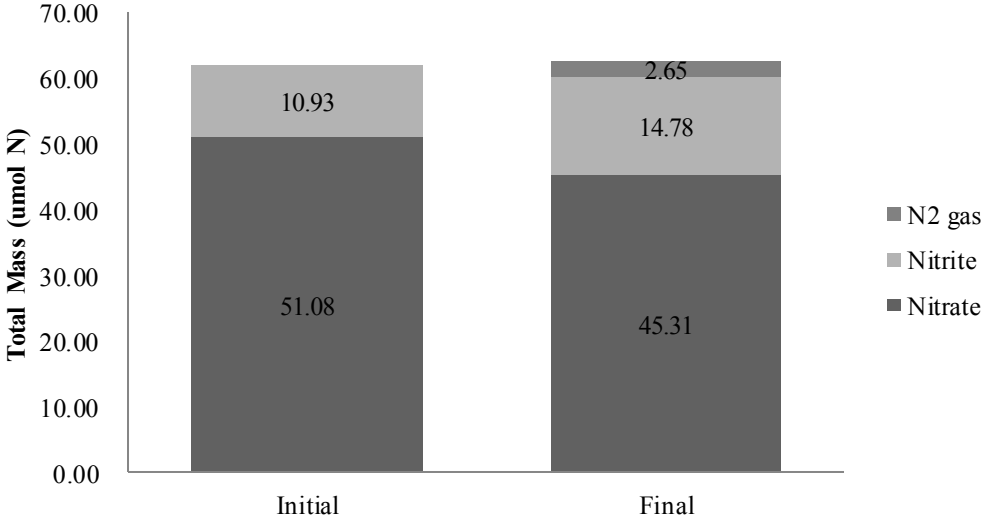
Run 2 （6 days）



Run 3 （6 days）



Run 4 （3 days）



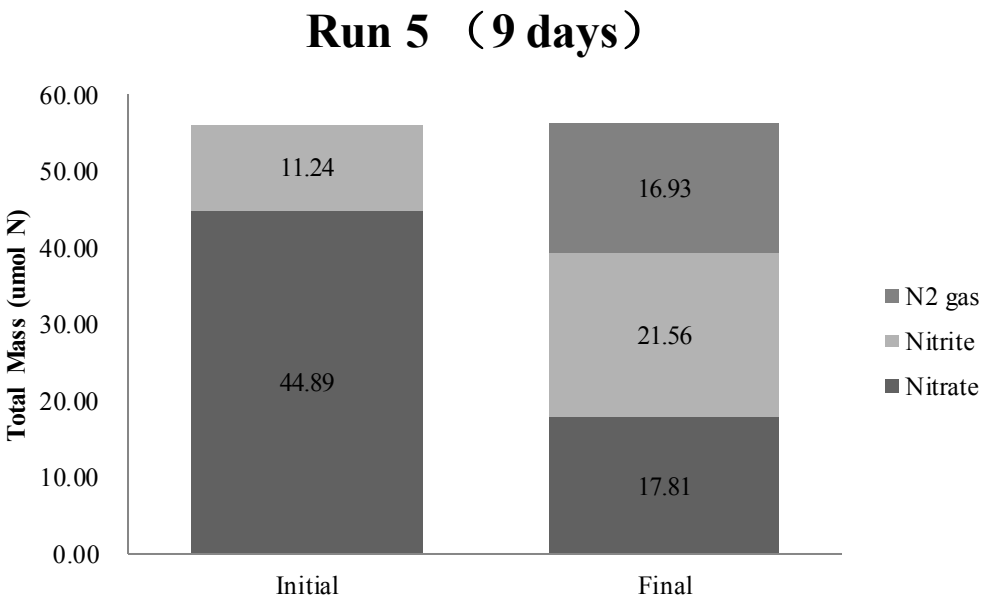


Figure 4.3 Mass balances in five tests.

4.3 Kinetics Test

4.3.1 Estimation of μ_{max} (the maximum growth rate)

Figure 4.4 presents the biomass (OD) data for the two batch growth experiments used to estimate μ_{max}. After a lag period of 1 day, biomass grew exponentially for about 5 days. μ_{max} was computed from the integrated equation 4.1:

$$ln\left(\frac{Xt}{X}\right) = \mu_{max} * t \quad (4.1)$$

Where X is the OD at the start of the exponential growth, and Xt is the OD at time t within the period of exponential growth. For the Figure 4.4 shown, μ_{max} was set to the average of 0.121/day with standard deviation, 0.030/day. The μ_{max} value gives a doubling time of 5.7 days.

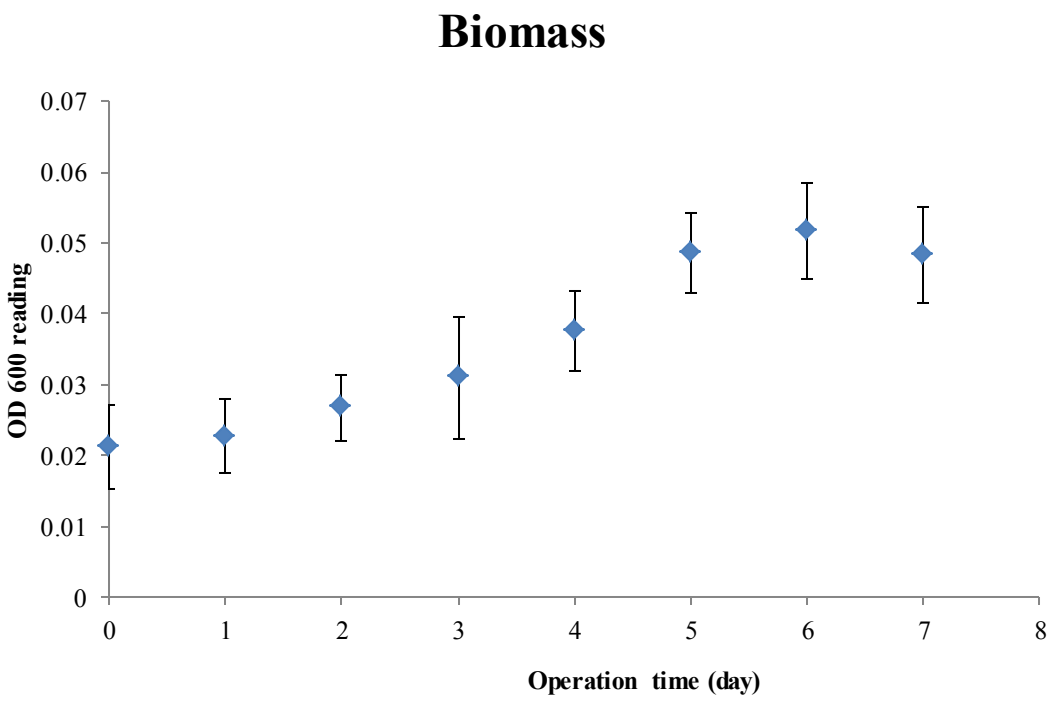


Figure 4.4 Biomass (OD) results at 37 °C in two batch growth experiments to estimate μ_{max}.

4.3.2 Estimating q_{max} (the maximum specific substrate utilization rate)

The maximum specific substrate utilization rate (q_{max}) was calculated using equation 4.2:

$$q_{max} = -\left(\frac{1}{Xa*V_L}\right)\left(\frac{d[CH_4]_L}{d_t}\right)\left(\frac{V_G}{R*T*K_H} + V_L\right) \quad (4.2)$$

Where V_L is the liquid volume (L), V_G is the headspace volume (L), R is the universal gas constant (0.082 L-atm/mol-K), and T is temperature in Kelvin, and K_H is the Henry's Law constant in mol/L-atm.

To obtain a reliable q_{max} value directly, first, the CH_4 concentration in the system must be significantly greater than the Ks value so that the (S/(Ks+S)) term can be simplified to 1. In addition, net biomass growth should be small for the duration of the experiment so that biomass term, Xa, can be set to as a constant value. To meet these criteria, satisfied the rate of CH_4 utilization in a batch was non growth experiment.

The q_{max} experiments were conducted with 0.3L of liquid volume (V_L) and 0.2 L of gas phase (V_G). The values for the other constants in equation 4.2 are R =0.08205 (L*atm/mol*K), T =310.3 K (37 °C), and K_H=1.6*10^{-3} mol/L*atm at 37 °C. Experimental (d[CH_4]L/dt) data at various biomass concentrations were shown in Figure 4.5. CH_4 concentration is expressed as liquid-phase concentration in mmol/L. Biomass concentrations are 0.021, 0.0245, 0.0615, and 0.0405 as OD (600) for panels a, b, c, d respectively.

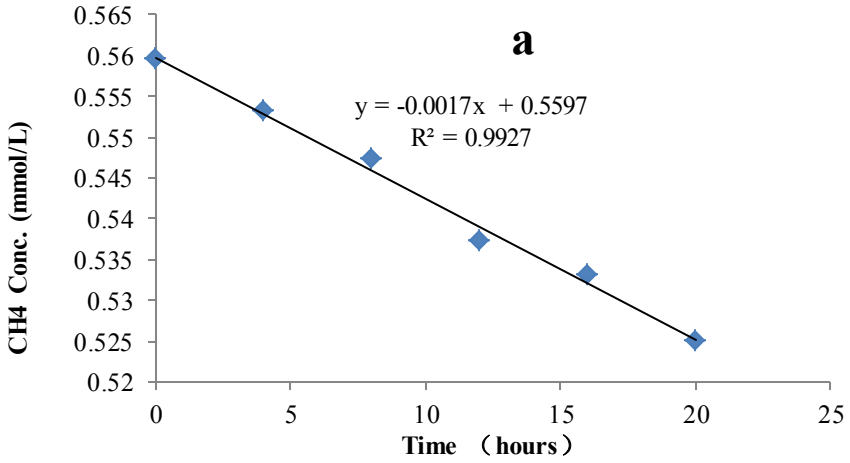
The CH_4 concentration declined steadily in each experiment. Linear regression was

performed to obtain the slopes and R^2 values shown in Figure 4.5 and the relevant values were tabulated in Table 4.1. The normalized substrate-consumption rates were computed using equation 4.2, in which Xa values are the mean biomass concentrations listed in Table 4.1, $(d[CH_4]_L/dt)$ values are the slopes in Figure 4.5. The mean q_{max} in Table 1 is 1.63 ± 0.85 mg COD/mg cells-day.

Table 4.1 Data for estimating q_{max} for different biomass levels.

Bottles	Mean biomass conc. (OD 600 reading)	Mean biomass conc. (mg cells/L)	CH_4 Consumption rate (mmol CH_4/L-h)	q_{max} (mmol CH_4/mg cells-day)	q_{max} (mg COD/mg cells-day)
a	0.021	31.86	0.0017	0.0222	1.42
b	0.0245	20.48	0.0022	0.0448	2.87
c	0.0615	77.73	0.0029	0.0155	0.99
d	0.0405	45.24	0.0021	0.0193	1.24

- Mean = 0.0254 ± 0.011 mmol CH_4/mg cells-day = 1.63 ± 0.85 mg COD/mg cells-day



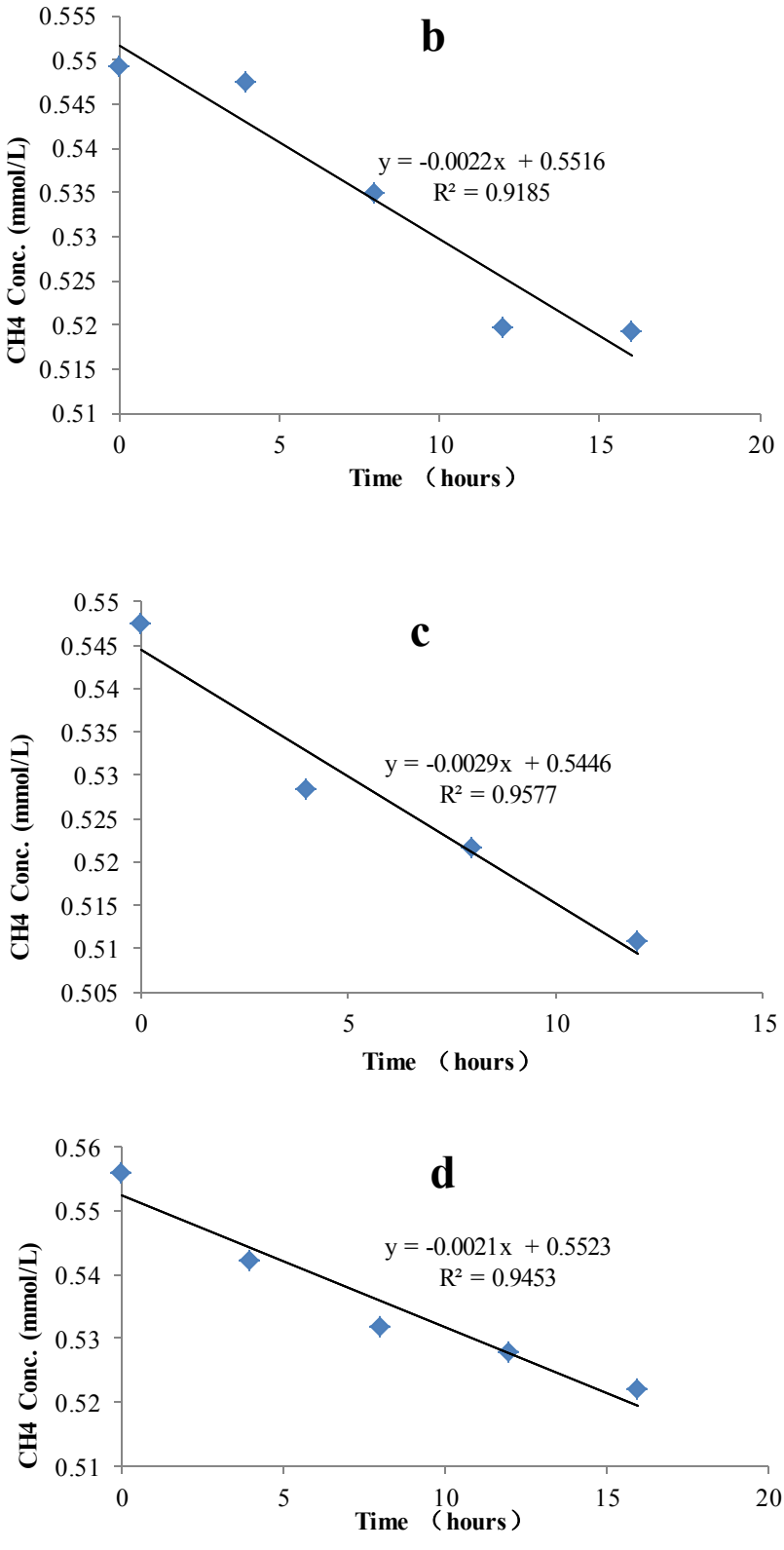


Figure 4.5 CH_4 consumptions for different biomass concentrations

4.3.3 Estimating Y (growth yields)

Using experimental μ_{max} and q_{max} values 0.121/day and 1.63 mg COD/mg cells-day., respectively, the yield can be computed according to $Y = \mu_{max}/q_{max}$, or Y =0.121 (1/day)/1.63 (mmol CH_4/g cells-day) = 0.074 mg cells/mg COD.

4.3.4 Estimating b (Endogenous decay constant)

Endogenous decay includes the oxidation of biomass to support cell maintenance. To obtain the endogenous decay constant experimentally, biomass concentrations of 0.049, 0.042, 0.084, 0.112 (OD units) were grown under non-limiting CH_4 conditions. These OD values represent biomass taken from the exponential growth phase.

To estimate b from the decay results, the synthesis terms were dropped in equation 4.3

$$\frac{dX_a}{dt} = -b * X_a \quad (4.3)$$

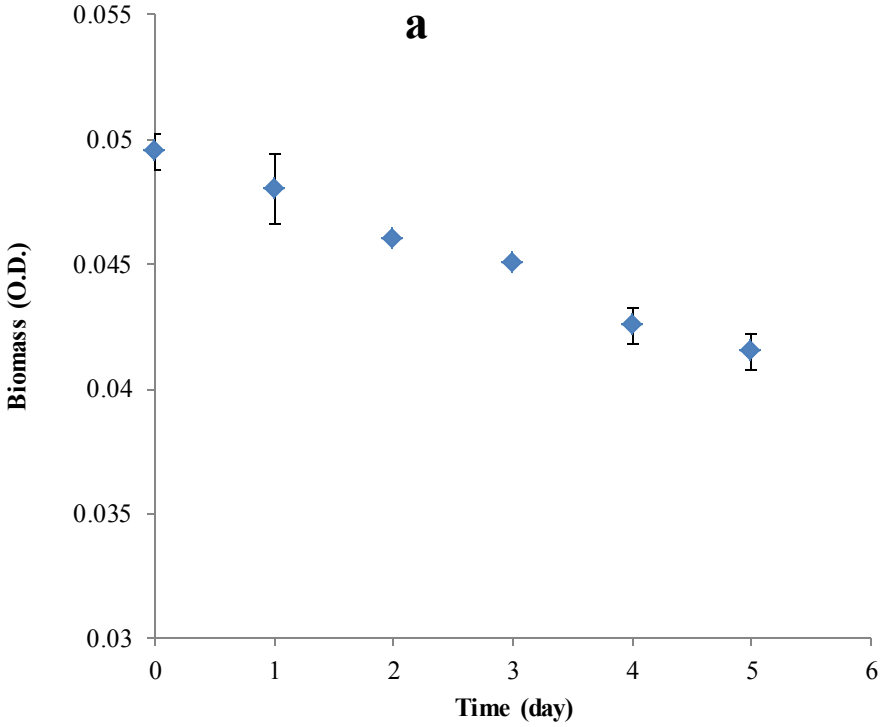
Because no CH_4 was present in the beginning of the experiment, the mass balance simplifies to Integrating equation 4.3 gives

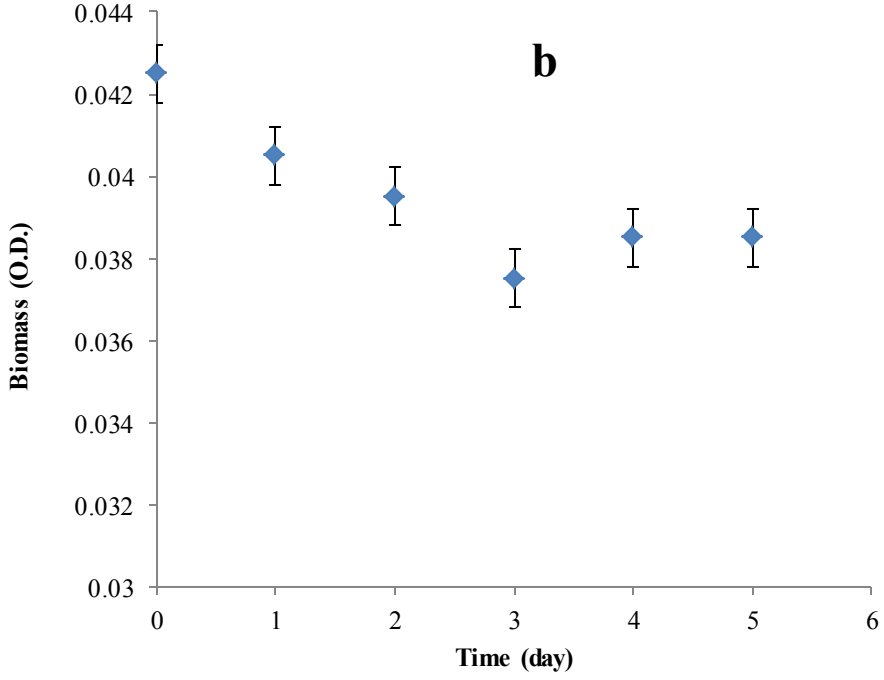
$$\ln\frac{X_0}{X_t} = b * t \quad (4.4)$$

Where Xo is the initial Xa concentration and Xt is the Xa value at time t of the decay experiment.

Figure 4.6 showed the experimental results from four group's decay experiments. When no CH_4 was present in the growth tubes, exponentially grown bacteria cells decayed over time. The b values were computed by equation 4.4. The average b value is

0.03 ± 0.002 /day.


a
0.055
0.05
0.045
0.04
0.035
0.03
Biomass (O.D.)
0
1
2
3
4
5
6
Time (day)


b
0.044
0.042
0.04
0.038
0.036
0.034
0.032
0.03
Biomass (O.D.)
0
1
2
3
4
5
6
Time (day)

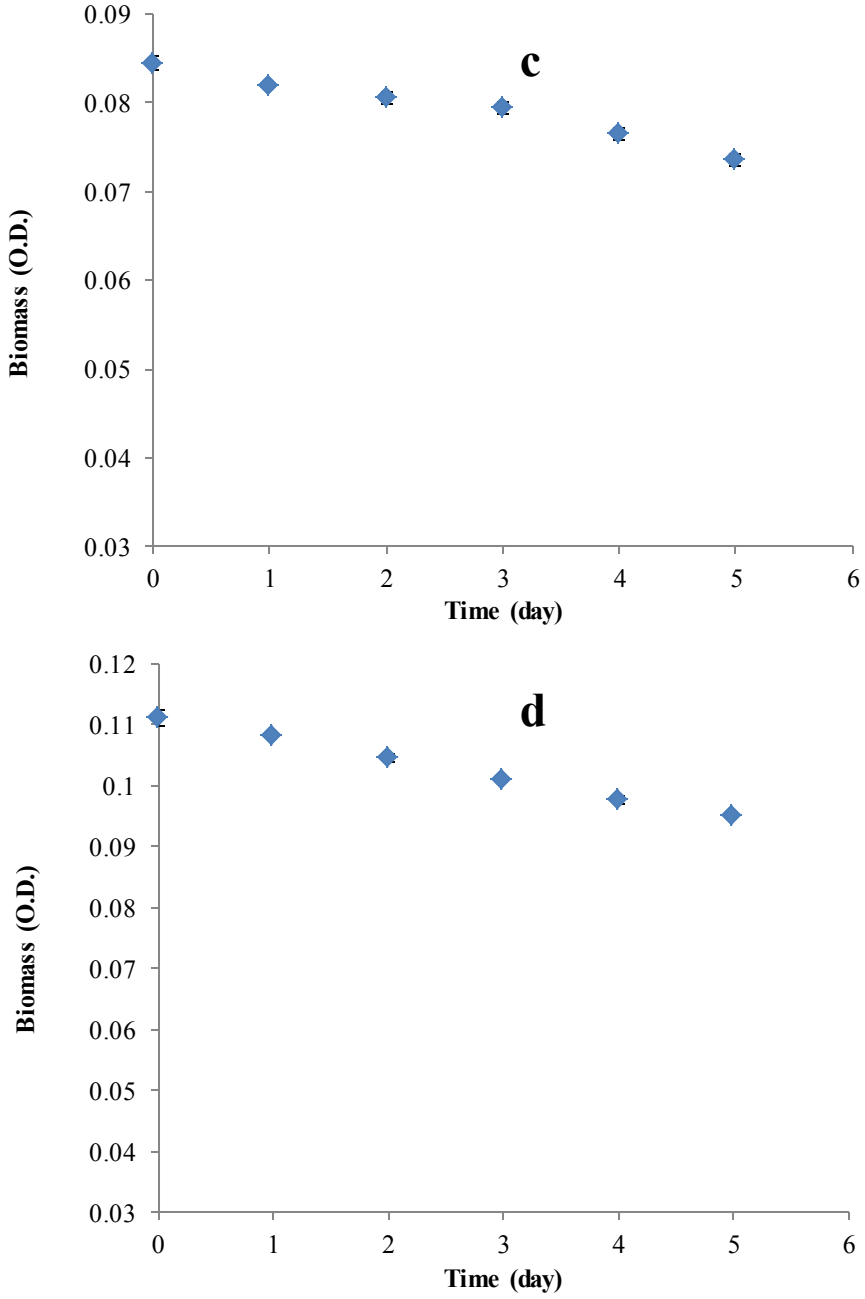
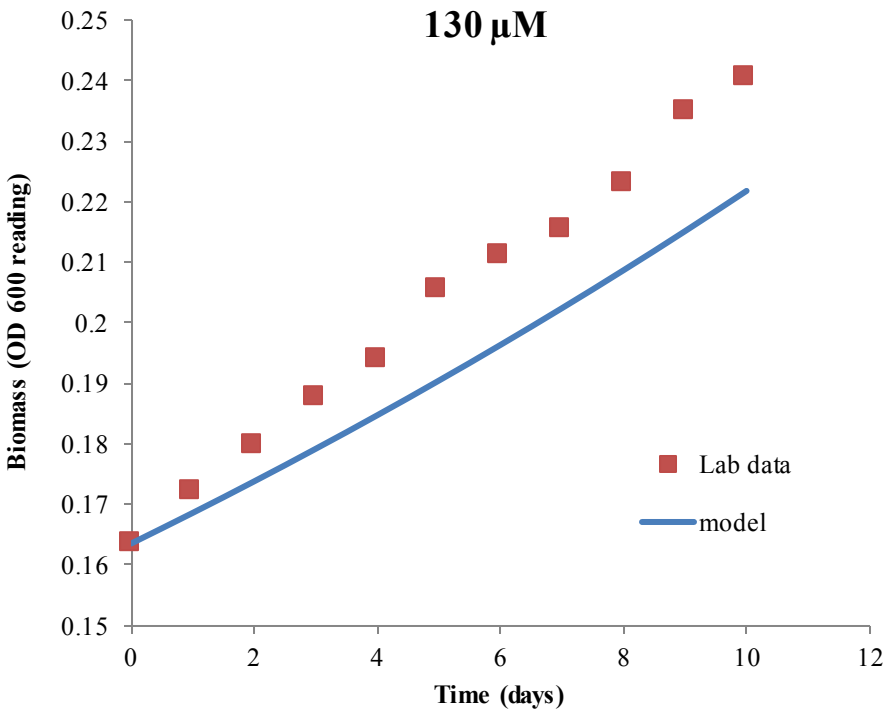


Figure 4.6 Biomass concentrations during four groups endogenous decay experiment.

4.3.5 Estimating Ks

To find Ks, batch growth experiments were conducted under conditions in which CH_4 rate was limiting. Because μ_{max}, q_{max}, Y and b were already estimated from the preceding experiments, Ks was found by fitting the experimental data from the Ks experiments into model simulations

Three experiments having initial CH_4 concentrations of 130, 119, and 90 µM were performed. Using equation 3.1 and 3.2, biomass growth in each bottle was simulated. Figure 4.7 compares the experimental results with model simulations for biomass growth. Initial biomass concentration (OD 600 reading) is very similar for all three experiments (a: 0.164, b: 0.159, c: 0.154) Figure 4.7 indicated that 90 µM CH_4 is the best fit for the model.



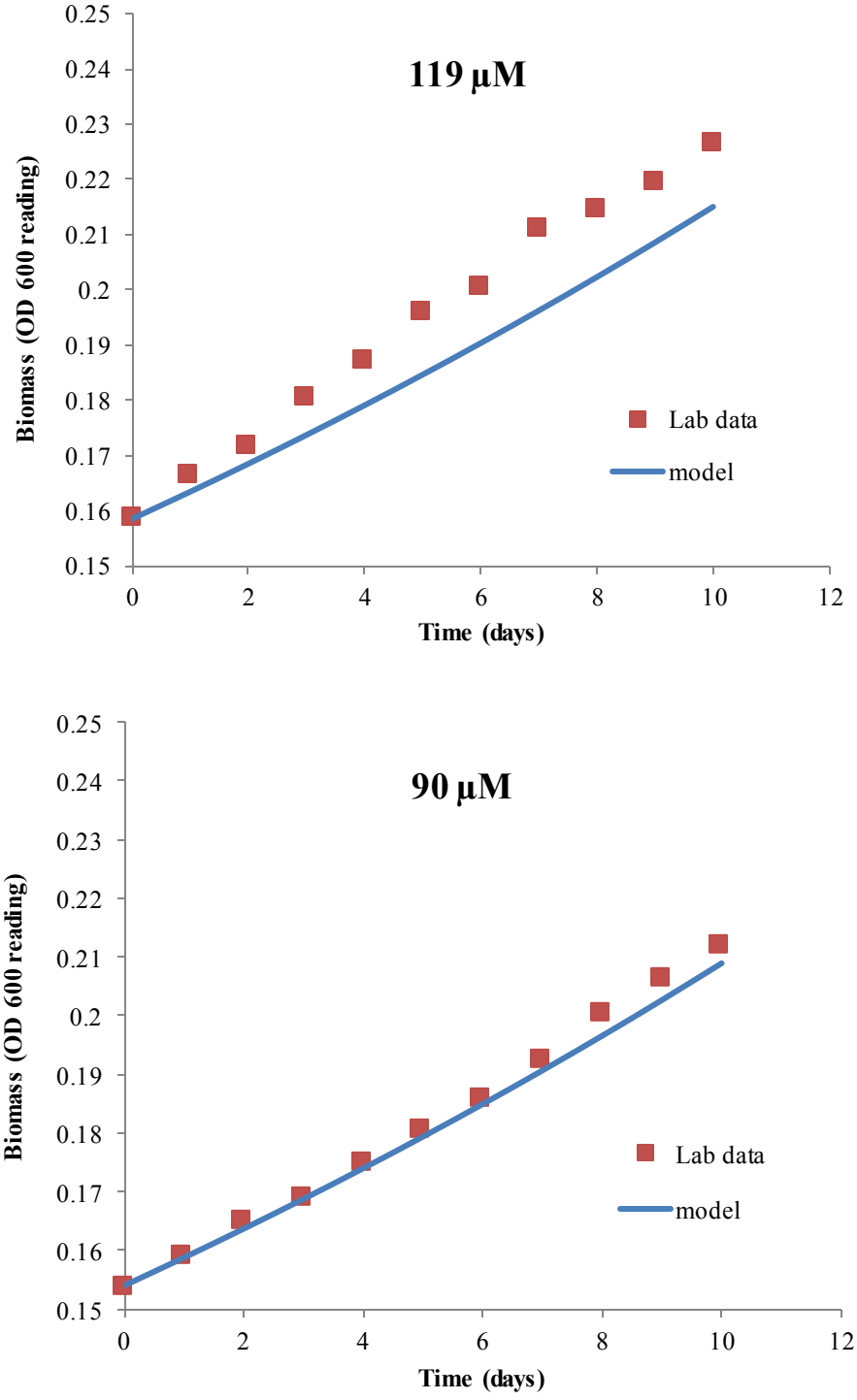


Figure 4.7 Model simulations (lines) and experimental data (symbols) for Biomass growth in three Ks experiments.

Figure 4.8 showed the relationship between biomass growth rate and dissolved methane concentration. By using a common Ks value of 85μM CH_4 all experiments' growth rate can fit in the model simulations. These good fits in the figure 4.8 can indicate that Ks value is 85μM CH_4.

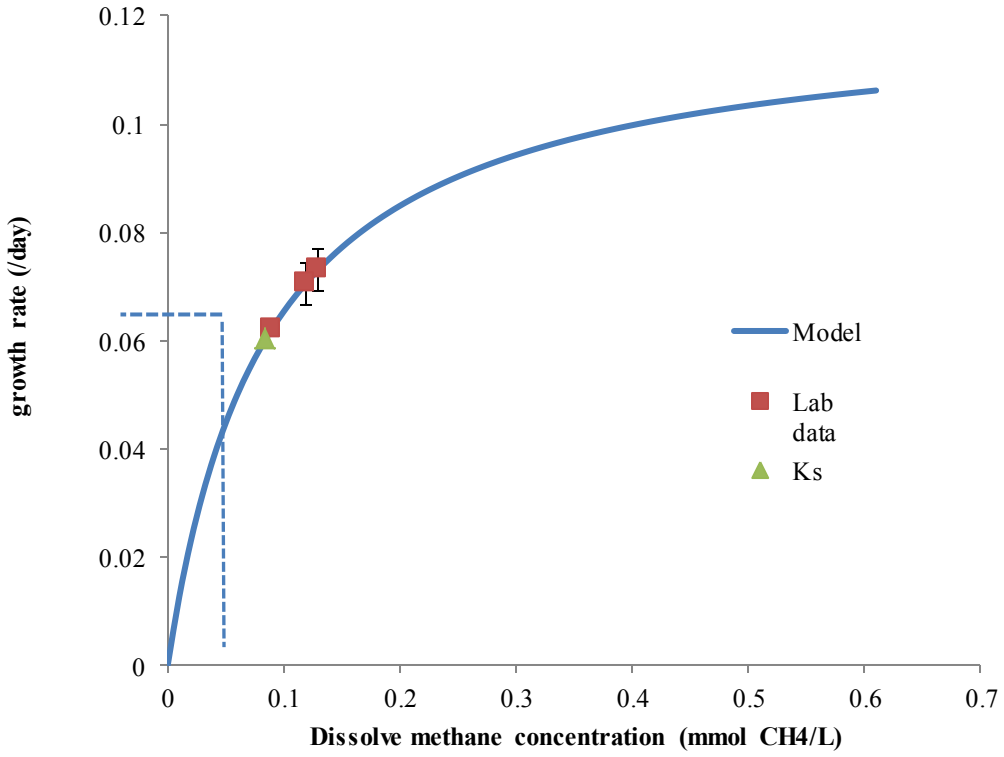


Figure 4.8 the biomass growth rate with different methane concentration.

4.4 Discussion

4.4.1 Comparison of denitrification electron donors

Table 4.2 and 4.3 summarize the results of characterization studies, including denitrification rates, and growth kinetics for different carbon sources found in literature as well as tested in this study.

In the heterotrophic biological denitrification part, methanol has been the industry standard for wastewater denitrification due to historically low cost, favorable kinetics, and low cell yield. There is a wide range for the denitrification rates for methanol, since several studies have been done on this compound. This variability is in part due to the different approaches uses to conduct the test (e.g. batch versus continuous), or for the sludge used (mixed activated sludge, acclimated or not, and pure culture).

Table 4.2 has shown that acetate was the most effective as carbon source and produced higher removal rates than methanol, ethanol , glucose and methane. Tam at al., (2002) concluded that the results could be explained biochemically. The glycolytic pathway and tricarboxylic acid (TCA) cycle are the two metabolic pathways for utilizing organic substrate as sources of energy and carbon in most organisms. Acetyl Co-A, which is easily formed from acetic acid or acetate is the key compound of these pathways; as a result, sodium acetate is a directly utilizable substrate which is more readily metabolizable than methanol, acetate, methane. As with acetate, ethanol is easily converted by the bacterial cell into Acetyl Co-A, before entering the TCA cycle, and in several studies was found to efficiently be available for denitrification with higher rate than methanol (Mokhayeri et al. 2006, Nichols et al. 2007). The lower rates associated

with glucose are most probably associated to the more complex degradation pathway thought which bacteria derive energy from sugars. Also Cokgor and colleagues (1998) found that the use of glucose in respirometric tests gives results difficult to interpreter and attributed this fact to the complex sequence of biochemical reaction this compound undergoes.

On the autotrophic biological denitrification side, elemental sulfur was studied most extensively mainly because of its low price, high sulfur content to mass ratio among the reduced sulfur compounds, and ease of handling (Hashimoto et al. 1987). Hydrogen is also a promising electron donor for denitrification because of its high selectivity for nitrate removal and the lack of a harmful by-product (Chang et al. 1999).

Based on the maximum specific growth rate (μ_{max}), Table 4.2 and Table 4.3 also show that the maximum specific growth rates for other carbon sources (in 1/day) are 0.5-2 for methanol, 1.89 for ethanol, 2.5 for acetate, 0.6-1.3 for hydrogen, 2.6-2.8 for sulfur. In Comparisons, the bacterium in this study is very slow growing, because its experimental μ_{max} (0.121/day) is at the lower end of the maximum range. This comparison of maximum specific growth rates indicates that the AMO denitrifying bacteria which are using nitrate may not compete well with other methanogens in general, when none of these groups are limited by its electron acceptor.

The substrate maximum utilization rate of AMO denitrifying bacteria is also relatively low compared to other groups. The value (1.63 mg COD/mg cells-d) is about half the lowest q_{max} (3.2 mg COD/mg VSS-d) reported for other groups, which indicates that the ANME-D bacteria have a clear disadvantage in utilizing methane as substrate efficiently.

A typical endogenous-decay coefficient for anaerobic microorganisms is 0.02/day (Rittmann, 2001). This value is a bit smaller than our experimental b value, 0.03/day. The similar b value makes ANME-D bacteria suited for survival in CH_4-limited environment.

Table 4.2 Summary of kinetic rate of heterotrophic biological denitrification.

Carbon source	Growth yields Y		Substrate maximum utilization rate q_{max}		Biomass maximum growth rate	Endogenous decay constant	Half saturation constant		Reference
	$(\frac{mg\ biomass}{mg\ NO_3-N})$	$(\frac{mg\ biomass}{mg\ C\ substr.})$	$(\frac{mg\ NO_3-N}{mg\ biomass*d})$	$(\frac{mg\ C\ substr.}{mg\ biomass*d})$	μ_{max} (d^{-1})	b (d^{-1})	$(\frac{mg\ NO_3-N}{L})$	$(\frac{mg\ C\ substr.}{L})$	
Methane (anaerobic)		0.074 mg cells/mg COD		1.63 mg COD/mg cells*d	0.121	0.03		5.44 mg COD/L	This study
Methanol		0.23-0.25 mg VSS/mg COD		8 mg COD/mg VSS*d	2				(Christenson et al. 1994)
					0.52(10°C)-1.86 (20 °C)				(Stensel *et al.* 1973)
					0.4-0.5(13°C) 1.0 (19° C)				(Mokhayeri *et al.* 2006)
		0.4 mg VSS/mg COD		3.2 mg COD/mg VSS*d	1.28	0.04			(Dold et al. 2008)
	0.28 mg VSS/mg N	0.29 mg VSS/mg COD	0.145 mgN/mgVSS*d	4.14 mg COD/mg VSS*d	1.2 (20 °C)	0.06	5		(Onnis et al. 2008)
			0.0576 -0.0864 mg N/mg VSS*d						(Ramalingam *et al.* 2007)
		0.166-0.195 mg SS/mg COD		4.4-13.3 mg COD/mg SS*d	0.52-1.86			43.6-60.0 mg COD/L	(Stensel et al. 1973)
Ethanol	6.85 (15°C) mg VSS/mg N	0.25-0.28 mg VSS/mg COD	0.276 (15°C) mgN/mgVSS*d	7.56 mg COD/mg VSS*d	1.89 (15°C) -				(Christenson et al. 1994)
			0.23 mgN/mgVSS*d						(Peng et al. 2007)
		0.22 mg VSS/mg COD							(Hallin et al. 1996)
					1.2 (13°C) 3.5 (19 °C)				(Mokhayeri et al. 2006)

Acetate			0.254 mg N/mg VSS*d						(Tam et al. 1992)
		0.66 mg VSS/mg COD	0.0864 mgN/mg VSS•d	3.78 mg COD/mg VSS*d	2.5		26 mg N/L		(Kujawa & Klapwijk 1999)
		0.35 mg VSS/mg COD	0.326 mgN/mg VSS•d						(Onnis et al. 2008)
			0.288 mgN/mg VSS•d						(Peng et al. 2007)
	2.7 mg VSS/mg N								(Soares et al. 2000)
Glucose		0.38 mg VSS/mg COD							(Muller et al. 2003)
			0.0912 mg N/mgVSS•d						(Prentice 2007)
	0.7 mg SS/mg N	0.33 mg SS/mg glucose					83 mg N/L	18.57mg glucose/L	(Beccari et al. 1983)

Table 4.3 Summary of kinetic rate of autotrophic biological denitrification.

Substrate	Growth yields Y		Substrate maximum utilization rate q_{max}		Biomass maximum growth rate	Endogenous decay constant	Half saturation constant		Reference
	$(\frac{mg\ biomass}{mg\ NO_3-N})$	$(\frac{mg\ biomass}{mg\ C\ substr.})$	$(\frac{mg\ NO_3-N}{mg\ biomass*d})$	$(\frac{mg\ \ substr.}{mg\ biomass*d})$	μ_{max} (d^{-1})	b (d^{-1})	$(\frac{mg\ NO_3-N}{L})$	$(\frac{mg\ C\ substr.}{L})$	
Hydrogen		0.2 mg COD/mg COD		5 mg COD/mg COD*d	1.0	0.05			(Rittmann & McCarty, 2001)
	2.1-2.3 mg cells/mg N		0.56 mg N/mg cells * d		1.2-1.3		40 mg N/L	0.208 mg COD/L	(Robinson et al. 1984)
			0.38–0.74 mg N/mg VSS*d						(Rezania et al. 2005)
	0.43 mg VSS/mg N								(Park & Yoo 2009)
	0.27 mg VSS/mg N		0.286 mg N/mg VSS*d		0.08				(Barlindhaug & Odegaard 1996)
	1.08 mg VSS/mg N		0.567 mg N/mg VSS*d		0.61				(Aesoy & Odegaard 1994)
Sulfur	0.4-0.5 mg biomass/mg N	0.3-0.4 mg VSS/mg substrate	5.76-7.2 mg N/mg biomass*d	7.2-9.6 mg substrate/mg VSS*d	2.88		3-10		(Oh et al. 2000)
			0.147 mg N/mg VSS*d						(Moraes et al. 2012)
	0.129 mg cells/mg N		0.01872 mg N/mg cells*d		2.64		0.2 mg N/L		(Gunter et al. 1985)
Fe					0.054-0.090				(Su and Puls, 2007)
Fe^{2+}					0.064				(Van and Wang,1995)

Fe^{2+}		7.47 mg VSS/mg COD			0.011	0.3			(Rolle et al., 2008)
$Fe(OH)_3$		14.27 mg VSS/mg COD					0.5		(Rolle et al., 2008)
Mn^{2+}		3.67 mg VSS/mg COD			0.008	0.001			(Rolle et al., 2008)
MnO_2		5.8 mg VSS/mg COD					0.5		(Rolle et al., 2008)

4.4.2 Comparison with other denitrification processes using methane as external carbon source

A compilation of denitrification rates obtained by different researchers on methane denitrification is shown in Table 4.4. It can be seen that the aerobic methane denitrification provide higher rates than both anaerobic methane denitrification since the anaerobic process was accomplished by very slow-growing microorganisms. However, the aerobic methane denitrification's rates are still lower than typical denitrification rates with methanol as the carbon source. The main reason is aerobic methane denitrification is fundamentally different from the methanol denitrification process. In the latter process, the reduction of nitrate and oxidation of methane (or methanol) are directly interlinked and dependent on each other, whereas in the former process, methane oxidation is not dependent on the reduction of nitrate and the occurrence of denitrification does not yield any specific benefits for the methane-oxidizing microbial population (Oskar, 2007). Another problem with aerobic methane denitrification is that it requires methane and oxygen to be supplied simultaneously to the microbial culture in potentially flammable mixtures. Methane is a greenhouse gas and it is therefore also very important that no methane is emitted to the atmosphere in this process (Waki et al. 2005)

In the field of anaerobic methane denitrification, Table 4.4 also showed that anaerobic methane oxidation coupled to nitrite has much higher denitrification rates than which coupled to nitrate. This indicates that under anaerobic conditions the methanotrophs which utilize nitrite are more competitive than the group which utilize nitrate. Anaerobic methane denitrification will have several advantages over aerobic

denitrificaition for applications in environmental engineering, both because it possesses a much higher efficiency in terms of C/N ratio and it does not require the presence of oxygen. However, based on other researchers' and this study's results, this process is very slow, which might limit its applicability for engineering purposes. More research is needed to clarify the mechanisms and the microbial community involved.

Table 4.4 Three types of methane denitrification rate.

Carbon source	Nitrate removal rate		References
	mg N/L*d	mg N/g VSS*d	
CH_4-NO_2 (anaerobic)	33.5-37.8		(C. Kampman et al. 2012)
	15.4		(Francisca et al. 2011)
CH_4-NO_3 (aerobic)	49.9	600 (max)	(Thalasso et al. 1997)
	259.2		(Rajapakse and Scutt 1999)
		16.3	(Khin and Annachhatre 2004a)
	60		(Waki et al. 2005)
	49.9	600 (max)	(Thalasso et al. 1997)
CH_4-NO_3 (anaerobic)	7.5	600	This study

4.4.3 The discussion of limiting factors

Methane is a really easy limiting factor of denitrification process for its low solubility in water, and especially previous studies of anaerobic methane oxidation indicated that high methane pressure could increase biologic activity (Katja Nauhaus, 2002; Zhang et al., 2010). The low Ks value of 85 μM CH_4 indicated that methane is a limiting factor of AMO denitirification process at atmospheric pressure from this study.

In addition , the AMO bacteria activity decrease or stagnation may be caused by the absence of growth factors (Ettwig et al., 2008). To enrich AMO bacteria and limit

other heterotrophic bacteria, there was no organic matter added into the present medium, which may limit the growth of AMO bacteria in the later period for growth factors deficiency. A previous study had focused on the problem and tried to add sewage treatment effluent as a source of potential growth factors, but did not draw a clear conclusion (Kampman et al., 2012). Further research should focus on the culture medium optimization.

4.5 References

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5. Conclusions

5.1 Summary of Results

In this study several experiments were operated to incubate AMO denitrifying bacteria and estimate the kinetic parameters of AMO coupled to nitrate. Together, these experiments show unambiguously that methane can be oxidized anaerobically in this system, and that this oxidation is coupled to nitrate denitrification. A kinetic model for AMO coupled to nitrate denitrification process was established. Several key kinetic parameters for n-damo bacteria were identified: maximum specific growth rate (μ_{max}) = 0.121/day, maximum substrate consumption rate (q_{max}) = 1.63 mg COD/mg cells-day, true yield (Y) = 0.074 mg cells/mg COD, half maximum-rate substrate concentration (K_s) = 85 μM CH_4, and endogenous decay rate (b) = 0.03/day. AMO denitrifying bacteria could offer a possible solution to counteract world-wide increases in methane production associated with intensive agriculture. With biomarkers and probes for the responsible microorganisms now available, this possibility can be addressed. However, the low growth rate indicate that the AMO denitrifying bacteria are slow growers, which means it may be a very time consuming process to cultivate enough biomass to achieve denitrification at desired rates. In addition，methane is a limiting factor of AMO coupled to nitrate process, probably due to its low solubility in liquid phase. This study firstly reported kinetic parameters for denitrifying AMO microorganisms, which will substantially help understand methane-utilized denitrification in natural systems in one hand. In the other hand, this new finding can catalyze the development of innovative denitrification processes using methane gas.

5.2 Recommendations

Based on the results of this research，several recommendations are provided for the future studies:

- Isolated genomic DNA from the biomass in the enrichment culture to determine the phylogenetic identity of the members of this consortium.
- To enrich AMO bacteria and limit other heterotrophic bacteria, there was no organic matter added into the present medium, which may limit the growth of AMO bacteria in the later period for growth factors deficiency. Further research should focus on the culture medium optimization.
- Further research should focus on systems with better biomass retention such as membrane bioreactors, reactors with granular sludge or biofilms to increase the volumetric consumption rates to the desired values.

6. Appendix

6.1 Photographs of Experimental Set-up



A.1 Serum bottle reactor



A.2 Serum bottles in the shaker



A.3 spectrophotometer for biomass measurement



A.4 Centrifuge



A.5 Anaerobic Chamber used to transfer bacteria