Role of microbial diversity in controlling greenhouse gas emissions from conserved forages

by

Maheen Mahmood

B.S., University of Engineering and Technology, Pakistan, 2016

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Civil Engineering
Carl R. Ice College of Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Approved by:
Major Professor
Dr. Jeongdae Im

Copyright

Abstract

Forages are plant materials utilized by grazing livestock. Forages are preserved to provide year-round availability of nutritious feed for livestock and typically conserved as hay (20% moisture content) or silage/haylage/baleage (40-70% moisture content). Silage undergoes natural fermentation process and anaerobic condition is the first and foremost requirement for the process. Under anaerobic condition, conserved forages may serve as an ideal habitat not only for fermenters but also for other diverse microbial groups such as methanogens and denitrifiers, which are involved in the production and reduction of greenhouse gases (GHGs), i.e., methane (CH_4) and nitrous oxide (N_2O). A laboratory study was conducted to examine the GHG production from alfalfa in the absence of oxygen (O_2). The results showed that 2.2 μmol of CH_4 per $g_{\text{-forage}}$ and 13.0 μmol of N_2O per $g_{\text{-forage}}$ were produced, which corresponds to 122.2 and 8,581.2 μg CO_2 eq per $g_{\text{-forage}}$., respectively. Based on the annual silage production volume reported by USDA, the N_2O emission potential from forage conservation process was estimated to be 0.3 million metric tons CO_2 equivalent per year, which ranks forage conservation as the third most important yet unaccounted source of N_2O emissions in the agricultural sector. In order to further validate the presence of these microorganisms in forages, PCR amplification was performed using primers targeting microbial genes of interest, especially those associated with denitrification and methane cycle in addition to bacterial 16S rRNA gene. PCR amplification results validated the presence of functional genetic markers for methanotrophs and denitrifiers. The gene marker for methanotrophs (pmoA) was detected prior to the incubation and on the outside of the haybales post two months incubation but disappeared from the inside. The detection of methanogens on the inside of haybale sample after two months of incubation suggested a microbial community shift inside the haybales.

The second objective of this study was targeted towards optimizing the protocol for the extraction of microbial DNA from silages as it was hypothesized that different DNA extraction protocols would result in different microbial DNA to total (microbial + plant) DNA ratio. Hence, we employed mechanical and enzymatic cell lysis procedures.

Table of Contents

List of Figures

List of Tables

References

Acknowledgements

I would like to acknowledge the support and guidance of my major professor Dr. Jeongdae Im, throughout this journey. I have received tremendous amount of inspiration and knowledge from him during the course of my master's degree. None of the work in the thesis would have been possible without his sound guidance and I would not have come this far without his help.

I would like to express my sincere gratitude to Dr. Ganga Hettiarachchi for her encouragement and for providing me the opportunity to utilize her greenhouse space and facilitating me to continue my experiments. I want to thank Dr. Prathap Parameswaran for departing his immense knowledge and for inspiring me to become a better researcher. I also appreciate their support for serving as my committee members too.

I would like to thank our department head, Professor Dr. Mustaque Hossain, for trusting me to be their teaching assistant.

I appreciate my friends and colleagues for their support during my master's journey. Nida Ghouri, for being a constant support since I was a beginner in the field of biotechnology and otherwise, throughout my stay at K-State. Finally, I wish to thank my parents for who have enabled me to start this journey, supported and believed in me.

Dedication

To my mother, who has always believed in me and my father, in whom I have always believed.

Chapter 1- Role of Microbial Diversity in Forage Conservation

1.1 Introduction

1.1.1 Microbial diversity

The significance and impact of microorganism on our ecosystems cannot be overlooked since they have been shaping our planet and its inhabitants for over 3.5 billion years.[1] They are considered as biogeochemical engines that continue to support all forms of life on Earth.

Microorganisms are major drivers of the Earth's carbon cycle. Nitrogen-fixation is another remarkable chemical feat achieved by microorganisms. Apart from these, they have been contributing to wide range of ecosystem functions. Since the microbial research has increased in the recent years hence it has yielded tremendous insight into the nature of the microbial communities, including their interactions and effects on the environment.

1.1.2 Forage conservation

Ensiled forage and haymaking have long been a fundamental link in the food chain as it serves as a stable feed with highly digestible nutrients compared with fresh crops. The ensiling of forage allows for year-round availability of nutritious and palatable feed while utilizing a smaller land base than grazing. Typically, forages are conserved in the form of hay, usually below 20% moisture (12-20% w/w), and silage with high moisture content (40-60% w/w) [1]. Hay is packaged (or baled) with twine or plastic net in rectangular or round bales for ease of handling, transport, and storage. Rectangular bales are as small as 36 cm high, 46 cm wide, and 102 cm long, but they are usually stored as stacks in a covered storage facility. Typical round bales are larger, 1.2–1.8 m in diameter and 1.2–1.7 m in length. For both shapes, anaerobic regions are inevitably created in the core area.

Silage is forage preserved by anaerobic storage, usually under conditions that encourage fermentation to provide lactic acid as natural preservative to inhibit undesirable microorganisms by lowering pH,[2][3] and to improve nutritional value.[4] Silages contain high moisture content (50~70%, w/w) and have several variations in preservation type including silage, haylage, and baleage primarily depending on their moisture levels and type of storage. Baleage is a round bale silage. Both haylage and silage can be preserved as baleage in which they are wrapped in a round bale with multiple layers of plastic excluding all the oxygen and creating partly or completely anaerobic conditions.[5]

1.1.3 Fermentation Process in Silage

Conservation of forage as silage involves natural fermentation process. Anaerobic condition is the first and foremost requirement for silage. Once anaerobic conditions are reached in the ensiled material, anaerobic microorganisms begin to grow. This process allows the natural microbes on the silage to ferment the natural sugars to organic acids such as lactic acid or acetic acid. The other microorganism such as yeast and molds have negative impact on silage as they compete with lactic acid bacteria for fermentable carbohydrates [6]. The principle of silage fermentation is to achieve a sufficient quantity of lactic acid to inhibit both the growth of undesirable microorganisms and the activity of plant catabolic enzymes, hence, maximizing nutrient preservation. Lactic acid will drop the pH to 4 or below within first week of ensiling. The period of active fermentation lasts from 7-21 days. [5]

1.1.4 Methane Production

Methanogenesis is an anaerobic respiration that uses oxidized carbon such as CO_2 as a terminal electron acceptor and produce ethane as a product. Fermentation allows breakdown of larger organic compounds and methanogenesis removes semi-final products such as small organics and

carbon dioxide.[7] Repeated spontaneous fire incidents have been reported from conserved forages. Investigations have revealed that certain changes can occur in improperly cured forage when it is tightly packed in a stack, and these changes may give rise to temporal variations and spontaneous combustion. It is commonly known that methane is the primary cause of these fire incidents however, limited research has been done on the presence of methanogens in silages.

1.1.5 Methane Consumption

On consumer side, methanotrophs, also known as methane oxidizing bacteria, utilize oxidized methane as a sole carbon source. Methane produced as a result of methanogenesis is conversely consumed by methanotrophs acting as a natural sink for CH_4. Because of the contribution of bacterial activity to global methane production and destruction, it becomes an important concern to understand these microorganisms involved and their responses to global environmental change.[8]

1.1.6 Nitrous Oxide Production

Nitrous oxide (N_2O) is the third largest contributor of GHG emissions to the atmosphere after CO_2 and CH_4.[9] Nitrous oxide is also a major source of ozone depleting nitric oxide (NO) and nitrogen dioxide (NO_2) in the stratosphere. [10] Research has revealed that mechanism for N_2O production has various players involved and stills needs to be understood.[11] Microorganisms share a major contribution in the production of N_2O through different processes.

1- In nitrification, it is proposed that N_2O is produced by ammonia oxidizers in two pathways: (i) reduction of nitrite catalyzed by nitrite reductase, and (ii) chemical decomposition of nitrite or intermediates of ammonia oxidation.

2- Nitrifier denitrification: It is the pathway of nitrification in which ammonia (NH_3) is oxidized to nitrite (NO_2^-) followed by the reduction of NO_2^- to nitric oxide (NO), nitrous oxide (N_2O) and

molecular nitrogen (N2). The transformations are carried out by autotrophic nitrifiers. Thus, nitrifier denitrification differs from coupled nitrification–denitrification, where denitrifiers reduce NO_2^- or nitrate (NO_3^-) that was produced by nitrifiers.[12]

3- Anaerobic ammonia oxidation: This is a biological process also named as “anammox”, short for anaerobic ammonium oxidation. The anammox reaction combines ammonium and nitrite directly into N_2 gas under anoxic conditions:

$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$

During annamox, oxidation of NH_4^+ occurs at the expense of NO_2^- produced by either heterotrophic NO_3^- reduction or aerobic ammonia oxidation, the first step of nitrification. [13]

4- Ammonia oxidizing bacteria: A few phylogenetically restricted groups of microorganisms are known to perform either of the two steps of nitrification (conversion of to NH_4^+ to NO_2^- and NO_2^- to NO_3^- , all of which are members of the domain Bacteria. Because they catalyze the first and rate-limiting step of nitrification, ammonia-oxidizing bacteria (AOB) have received considerable attention in a wide variety of habitats.

5- Ammonia oxidizing archaea: Recently, ammonia oxidizing archaea (AOA) have been known as the source of nitrous oxide production along with the release of methane. Considerable analogies between the activities of ammonia oxidizing bacteria (AOB) responsible for N_2O production and ammonia oxidizing archaea (AOA) propose that AOA contributes in generation of nitrous oxide too.[13][14][15]

6- Denitrification: Among all of them, denitrification is a sequence of reductive reactions, and is thought to occur mostly in anaerobic environments. For this reason, the potential of N_2O production from denitrification process is highest in anaerobic ecosystems.

1.1.6.1 Denitrification

Denitrification is the part of nitrogen cycle and a respiratory process which is mediated by denitrifying bacteria under anaerobic conditions. Denitrification leads to nitrogen loss from agroecosystems through emissions of N_2 and the potent greenhouse gas N_2O. In denitrification, oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. It is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water, thus completing the N-cycle. This removal of soluble nitrogen oxide from the biosphere is of great importance in agriculture, where it can account for significant losses of nitrogen fertilizer from soil.

This bacterial denitrification consists of four reaction steps: conversion of nitrate (NO_3^-) to nitrite (NO_2^-), NO_2^- to nitric oxide (NO), NO to nitrous oxide (N_2O) and N_2O to N_2. These four reactions are catalyzed by specific reductase enzymes encoded as nitrate reductase (NarG), nitrite reductase (NirK), nitric oxide reductase (NorB) and nitrous oxide reductase (NosZ).

The nitrite reductase is the key enzyme of this respiratory process since it catalyzes the reduction soluble nitrite into gas. [16]

1.1.7 Nitrous Oxide Consumption

Present greenhouse gas models presume that nitrous oxide to nitrogen reduction (i.e., the final step of the denitrification pathway) is the major attenuation process controlling N_2O flux to the atmosphere. Hence, mitigation of N_2O emissions to the atmosphere has been attributed exclusively to denitrifiers possessing NosZ, the enzyme system catalyzing N_2O to N_2 reduction.[17] But attempts to predict N_2O emissions based on denitrifier nosZ gene abundance and expression revealed an incongruity between the predicted and the actual N_2O emissions, suggesting the existence of an unaccounted N_2O sink.

According to the previous research, complete denitrifiers have been considered the key functional guild that controls N_2O emissions from soil to the atmosphere. But further analysis of the typical denitrifier nosZ gene demonstrates that this was an incomplete analysis and is insufficient to account for or accurately predict N_2O flux. Attempts to predict N_2O emissions based on de-nitrifier nosZ gene abundance and expression revealed an incongruity between the predicted and the actual N_2O emissions, suggesting the existence of an unaccounted N_2O sink.

Thus, the discovery of functional, atypical nosZ genes from Bacteria and Archaea from a variety of habitats, including agricultural soils, indicates that a much broader group of microorganisms contributes to N_2O turnover.[18] This heretofore unrecognized diversity broadens the understanding of the ecological controls of N_2O consumption and the contributions of microbes with atypical nosZ genes should be considered in monitoring regimes and future greenhouse gas flux models. All complete de-nitrifiers are facultative aerobes and represent a homogeneous group that switches from oxygen respiration to denitrification when soils become anaerobic. Research has shown that non denitrifying N_2O reducers with atypical NosZ are eco-physiologically more diverse and occupy a much broader range of habitats. Research findings indicate that microbial populations with atypical nosZ genes are potential contributors to N_2O reduction in soils and other habitats where N_2O sources (e.g., chemo denitrification, nitrification) exist. These findings further demonstrate that the combined contributions of both complete denitrifier and non-denitrifier N_2O reducers must be quantified to obtain a meaningful measure of the catalysts involved in N_2O reduction. Since previously applied molecular tools used to estimate nosZ gene activity were not comprehensive and miss the contributions of microbes carrying an atypical nosZ gene and this underestimated the actual activity. This research also accounts for understanding N_2O flux and nosZ gene activity.

1.1.7 Greenhouse gas emission potentials from forage conservation

Greenhouse gases are the main contributor to the global warming and climate change due to their absorption of infrared radiation from the earth's surface.[1] Although CO_2, one of the GHG constitutes majority of GHG emissions and can live in the atmosphere for hundreds of years, other non- CO_2 gases such as methane (CH_4) nitrous oxide (N_2O) also accounts for 25% of global GHGs emissions yet nitrous oxide (N_2O) is around a relatively short time. But it stays in the atmosphere longer than other short-lived climate pollutants like black carbon (which exists in the atmosphere for days) or methane (which is around for 12 years). It is also a major source of ozone-depleting nitric oxide and nitrogen dioxide in the stratosphere and is currently the single most important ozone-depleting substance.[19] Among others, agricultural activities are the largest anthropogenic source of N_2O comprising 79%.[1] The emission will increase as it is closely linked to the most basic human need – food, unless substantial efforts are made to reduce the environmental footprint of agriculture.

Since soils act as sources and sinks for greenhouse gases (GHG) such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) and both storage and emission capacities may be large, precise quantifications are needed to obtain reliable global budgets that are necessary for land-use management (agriculture, forestry), global change and for climate research.

1.1.8 Aims and Objectives

As state earlier, anaerobic region is inevitable in the core of silages, therefore, it was hypothesized that conserved forages can serve as ideal habitats for diverse microorganisms not only for fermenters but also those involved in the production of GHGs, e.g., methanogens and de-nitrifiers,

that are known to produce CH_4 and N_2O, respectively. This study is targeted towards following goals

(1) To provide a preliminary assessment of the contribution of forage conservation to GHG emissions
(2) To characterize phylogenetic and functional diversity of microbial community in conserved forages

1.2 Materials and Methods

1.2.1 Sample Collection

For preliminary experiments, four round bales from second cut alfalfa were prepared for laboratory and field experiment. The bales were transported on the day they were baled. One of the haybale was used to collect samples for laboratory experiments. For simulating silage, 20 g of forage samples were added in 160 ml serum bottles, and the moisture contents were adjusted to 40% and 60% (w/w) by adding sterilized deionized distilled water and gas production was monitored. In order to validate the laboratory results, a field study was carried out at the K-State Beef Stocker Unit. In the field experiments, three round bales were monitored for surface greenhouse gas fluxes once a month for four months using flow through chamber approach. Flow through chamber system is known for its advantage of obtaining high resolution greenhouse gas (GHG) emissions over other gas flux systems such as static chamber method that utilizes manual closure of chambers for a specified period of time. This includes repeated collection of air samples and results in increased workload and reduced sampling events. Eventually, underestimating the net emission of nitrous oxide [20]. Hence, a customized closed chamber with two gas ports was installed on the

surface of round hay bales, and CH_4-free argon controlled by a flowmeter was passed into the chamber at a rate of 5 mL/min. The chamber headspace was continuously mixed with a battery powered fan installed inside[21]. The outflow gas samples were collected, measured in the laboratory. The fluxes were calculated using the following equilibrium equation, $F = C \cdot q/A$, where F is emission flux (μg/m^2·hour), C is concentration (μmol/m^3), q is air flowrate (m^3/hour), and A is surface area (m^2). Three forage core samples were collected using a hay coring probe at two different depths and DNA was extracted. The optimized forage amendment selected from the laboratory experiments was applied to prepare three silage bags (2.5 m x 2.5 m) along with additional three bags as controls. Four gas bags were connected, apart from each other. Similar to the laboratory experiments, the gas bags were periodically replaced with new ones, and the gas composition and total volumes was monitored. The gas/forage samples were collected once in two weeks molecular biological analyses.

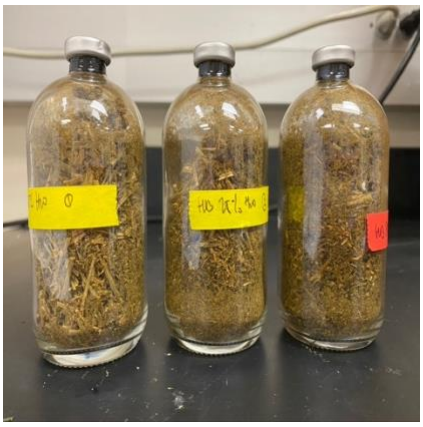

Figure 1 Haybale samples incubated in laboratory

Figure 2 Measurement of GHG flux from haybales

1.2.2 Gas Composition

For the gas composition analysis, Gas Chromatography (GC 7890A, Agilent Technologies) coupled with flame ionization detector and electron capture detector was used for the measurement of methane and nitrous oxide respectively. Gas sample of 100µL was injected for the confirmation of the CH_4 and N_2O in GC. GC-FID fitted with column J&W 123-1364 (60m x 320µm x 1.8µm) was used for CH_4 measurement under the following conditions with helium used as a carrier gas: split ratio 5:1, split injection rate 4.13/min, oven temperature program 100ºC, carrier gas flow rate He 3mL min^{-1} and CH_4 was detected at 2.739 minutes. GC-µECD fitted with Agilent 19091P column (30m x 320µmx 20µm) was used for N_2O measurement under following conditions: split ratio 20:1, split injection rate 86mL/min, oven temperature program 60ºC, carrier gas flow rate He 4.3 mL min^{-1} and N_2O was detected at 1.334 minutes.

1.2.3 PCR Amplification

PCR amplification was performed using primers targeting bacterial gene of interest, especially those associated with denitrification and methane cycle together with bacterial 16S rRNA gene. The primers for the amplification of targeted genes *nirK (nirKC1f/nirKC1r, nirKC2f/nirKC2r), nirS (cd8f/cd2R), norB (qnorB, cnorB), nosZ (nosZf/nosZr)*, bacterial *amoA (amoA1f/amoA2r)*, archaeal *amoA (arch-amoAf/arch-amoAr)* and archaeal *16S* were selected from previously designed sequences as mentioned in the references [22],[23],[24],[16][9] respectively. Primer sequences are mentioned in Table 1.

PCR amplifications from environmental samples including four field samples and one sample from laboratory incubation were performed in a total volume of 49µL containing 5µL of 1 X PCR reaction buffer, 1.5 µL of 50mM $MgCl_2$, 1µL of 10mM deoxyribonucleoside triphosphate(dNTP), 2.5µL of 10µM forward primer, 2.5 µL 10µM reverse primer, 0.20U/µL DNA Polymerase, 1µL of DNA template using Applied Biosystems MiniAmp PCR thermal cycler. The PCR cycle

parameters for all the targeted genes are listed in the table. The amplification products were analyzed by gel electrophoresis on 1% (weight/volume) electrophoresis grade gel in 20 ml TBE Buffer (Boehringer, Ingelheim, Germany), 2μl of 10,000X DNA staining mixed with 10ul PCR amplicon and 2μl of loading dye. For each well, 10μL sample was loaded.

1.2.4 Quantitative PCR

To quantify potential denitrifying microorganisms, methanogens, methanotrophs and bacterial 16S rRNA and their relative abundance in each sample, qPCR targeting specific genes as well as the bacterial 16S rRNA gene was intended to be performed, using primer pairs listed in the Table 1 and standards for each primer pair mentioned in Table 1 were designed.

For nirK(nirKC1F/nirKC1R) nucleotide sequence of *Ochrobactrum anthropi JCM 21032*,[25] nirKC2(nirKC2F/nirKC2R) nucleotide sequence of *Azospirillium lipoferum NBRC-1022290*, [26], nirS(cd8F/cd2R) sequence of *P. stutzeri ATTC 14405*, [23], *Ralstonia eutropha H16* for qnorB(qnorB2F/qnorB5R) [27], *Pseudomonas sp. strain G-179* for cnorB(cnorB2F/cnorB7R), [28],for bacterial 16S (1100F/1492R) *Lactobacillus delbrueckii subsp. bulgaricus strain LJJ* were downloaded from NCBI Database (https://www.ncbi.nlm.nih.gov) and binding location of each primer pair was located. The target DNA segment was trimmed from those locations and this synthesized DNA fragment was used to generate a standard curve of 10-fold dilution series. For nosZ genes (nosZI, nosZII), conserved regions were located by downloading multiple nucleotide sequences from NCBI database. The sequences were being imported and aligned into the MEGA4 software [29], where the alignment was manually checked and trimmed for conserved regions.[9]

1.3 Results

As shown in the graph, within 2 months of incubation, up to 2.2 μmol of CH_4 and 13.0 μmol of N_2O were produced, which corresponds to 122.2 and 8,581.2 μg CO_2 eq per $g_{\text{-forage}}$., respectively. The production rates of both gases were slower with lower moisture content (40%) but the total concentrations at the end were similar regardless of the moisture content. Nitrous oxide and methane were measured from the surface flux samples. Methane was not detectable but 0.66μmol N_2O/day/m_2 was measured. Functional genetic marker for methanotrophs pmoA was detected suggesting CH_4 was oxidized by methanotrophs.

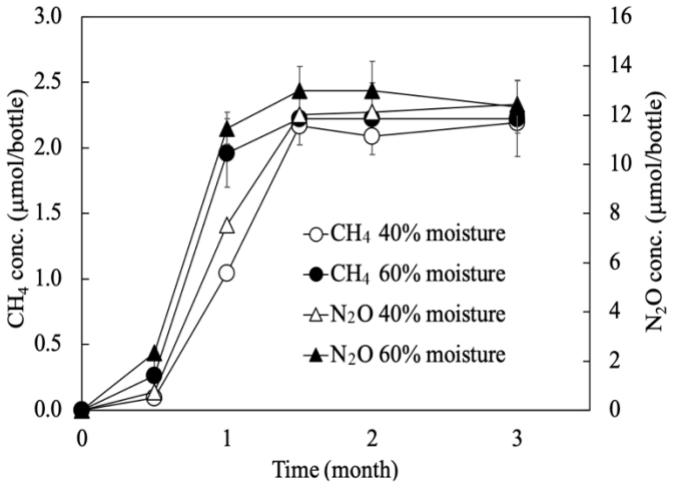

Figure 3 CH_4 and N_2O production from forages under anaerobic condition

In order to further validate the presence of these microbial community in forages, PCR amplification was performed using primers targeting bacterial gene of interest.

Methanogens can be monitored using genes and transcripts of *mcrA*, which encodes an enzyme that catalyses the final step in methanogenesis and it is the most frequently used biomarkers for the determination of methanogenic populations in environments.[30]

For CH4 oxidation, the enzyme responsible for the first step in the oxidation is known as methane monooxygenase (MMO). Two forms of this enzyme exist, a soluble enzyme complex (sMMO) and a membrane bound, particulate enzyme (pMMO).[31] The α subunit of pMMO is encoded by the *pmoA* gene. This is present in all known methanotrophs with the exception and is commonly used as a genetic marker for methanotrophs.

Denitrifying microorganisms can have different combinations of genes involved in the denitrification pathway.[32][33] The functional markers to investigate diversity of denitrifying bacteria were nirS,nirK, norB, nosZ genes.

	pmoA	***mmoX***	***mcrA***
Day 0	+	-	-
1 month (inside)	-	-	+
1 month (outside)	+	-	-
2 months (inside)	-	-	+
2 months (outside)	+	-	-
Laboratory	_	-	+

Table 1 PCR Result of functional gene markers for methane cycle

	nirK	**nirS**	**norB**	**nosZ**	**nrfA**
Day 0	-	-	-	-	-
1 month (inside)	+	-	+	+	-
1 month (outside)	-	-	-	-	-
2 months (inside)	+	-	+	+	-
2 months (outside)	-	-	-	-	-

Laboratory	+	-	+	+	+

Table 2 PCR Result of functional gene markers for denitrification cycle

+: detected **-: not detected**

1.4 Discussion

According to the reports of environmental protection agency (EPA) on the account of greenhouse gas emission potentials from agricultural activities, it is quite evident that these activities contribute directly to emissions of greenhouse gases through a variety of processes.[10] Methane, nitrous oxide and carbon dioxide are the primary greenhouse gases emitted by agricultural activities.

From the data of our gas production analysis from laboratory scale experiments, if we would assume that similar amount of methane and nitrous oxide would be produced from the total forage production in the US, the total emission potentials of CH_4 and N_2O would be 0.004 and 0.25 million metric tons (MMT) CO_2 equivalent.

According to aforementioned report, 0.20 million metric tons (MMT) CO_2 equivalent of nitrous oxide is being produced from field burning of agricultural residues. This ranks forage conservation as an unaccounted yet an important source of GHG emissions for nitrous oxide. Hence, analyzing greenhouse gas emissions from forages is the critical step to effectively managing them afterwards as GHG inventories are used by policy and decision makers to record GHG emissions, to form strategies and policies for climate change mitigation.

When CH_4 was measured from the surface flux samples, no significant concentration of methane was detected suggesting CH_4 may have been biologically oxidized by methanotrophs. Further, PCR amplification results confirmed the presence of the gene marker (*pmoA*) in the day-0 sample and the outer layer samples after one and two months of incubations, respectively. Further, *pmoA*

disappeared within one month of incubation in the inner layer, presumably in the absence of O2, suggesting that the methanotrophic activity was dependent on the substrate availability, i.e., CH4 and O2. Weak but detectable band of functional genetic marker for methanogens (mcrA) was detected from the inside of haybale sample. This was verified by the detection of genetic marker for methanogens on the inside of haybales. The functional genes responsible for the reduction of nitric oxide to nitrous oxide and nitrous oxide to nitrogen were also detected inside the core of haybales.

1.5 Recommendations

In order to replicate GHGs emissions on the laboratory scale, the research can be expanded further by constructing laboratory scale mini-silos and preparing silage from alfalfa. As silage is defined as the acidic and fermented product from an agricultural crop, there should be low terminal pH as the lower the pH, the more stable is the silage producing a hostile environment to inhibit the propagation of spoilage of microorganisms. However, pH alone is not a totally accurate monitor of silage fermentation.[34]. The basic idea is to customize a lab scale reactor that is capable for simulating forage conservation process under anaerobic conditions. Customized laboratory scale reactors can be made using acrylic cylinders and the top of the reactors can be covered with round acrylic glass disk with a port for sampling of gases. The vessel should be sealed properly to ensure no losses of the gases produced. Post ensiling temperature should maintained at 30°C [5]. In the preliminary experiments, a significant volume of CO_2 was produced, which may disturb microbial community unless properly released.[35] Gas bags (1 L capacity, SKC Inc. Eighty four, PA) should be connected to the sampling port installed in the headspace with the plastic tubing to collect additional gas and maintain atmospheric pressure in the headspace. The gas bags should be periodically replaced with new ones to characterize the temporal variations in gas composition

until the gas production stops. Whenever the gas bags have to be replaced, the total gas production volumes can be measured by water displacement method.

1.5.1 Chemical Treatments

Chlorate is known to inhibit microbial denitrification activity by blocking nitrate reductase. In a previous study conducted it was found that cattle drinking water containing as high as 100 mM sodium chlorate was fed for 24 hours to inhibit E. Coli O157[36]. In further studies, 0.1% of chlorate weight by weight of fresh forage [37] can be added to the reactors prior to ensiling. In addition to chlorate, silage inoculant can be added to help improve fermentation, retain nutrient content and enhance digestibility in ensiled forage. Low C/N ratio is one of the potential causes for N_2O accumulation during denitrification and acetate will be added as an external carbon source. Similar to the chlorate amendment, 0.1% of acetate weight by weight of fresh forage for corn will be amended along with the silage inoculants. Addition of silage inoculants is a common practice by farmers in the market[38]. These recommendations implemented with the lab scale reactors would facilitate us to confirm if denitrifiers are the main reason for greenhouse gas emission. If the results of chemical treatments with denitrifier inhibitors will indicate reduction in the concentration of nitrous oxide that will be an evidence of denitrifiers as the main source of N_2O production which is a major greenhouse gas and currently the single most dominant ozone-depleting substance as mentioned earlier. The key here, is to make sure fermentation conditions and nutritional value of forage remains intact. Therefore, samples will be analyzed for their nutritional values before and after the experiment has been terminated. The research will directly impact industrial development of sustainable agriculture practices.

Chapter 2 - Microbial DNA Extraction from Ensiled Forage

2.1 Introduction

As stated in the previous chapters, ensiling of forage is a global practice to provide nutritious feed to the livestock and microbiome associated with freshly harvested forage plays a critical role in the ensiling process. Once introduced into the silo, it was hypothesized in previous chapter that the diversity of microbiome shifts and in order to understand the microbial diversity in the silos, optimized protocol for the extraction of microbial DNA from forage is essential. Molecular techniques can revolutionize our understanding of the role of microorganisms in ensiling process and hence the objective of this study is to optimize the protocol for maximizing the extraction of microbial DNA from total forage DNA sample.

The DNA extraction has been eased by variety of commercial kits but it is challenging to select one when there is no specific kit available for extracting microbial DNA from plant. It was hypothesized that different DNA extraction protocols would result in different microbial DNA to total (microbial + plant) DNA ratio that may interfere with downstream application. Hence, employed mechanical and enzymatic cell lysis procedures were employed. It has been reported that complete lysis of bacterial cell wall is critical for optimum yield of DNA.[39] Lysis protocols include procedures that lead to physical and or enzymatic disruption of the microbial cell wall. It has been observed that extended lysis time and mechanical disruption can enhance nucleic acid yield.[40]

Qiagen DNeasy Power Soil Kit utilizes mechanical disruption (i.e., bead beating method) to break open the cells. Three different bead beating durations were examined to maximize the ratio and to analyze if longer and more vigorous bead beating procedures would increase the yield as suggested [41]. Some commercial kits utilize chemicals (e-g lysozyme and proteinase K) that promote lysis

at elevated temperatures. Qiagen blood and tissue kit utilizes chemical proteinase K for lysis. Different incubation times were tested to analyze if incubating for longer time will have accelerated effect on yield of DNA.

2.2 Extraction Protocols

In order to implement molecular technologies such as PCR it is essential to ensure that the extraction protocol employed obtains high quality DNA and yield of nucleic acid needed to achieve high quality sequences. [38] Samples should be frozen immediately to impede microbial activity. Microorganisms also invade into the interior of plant cells where the concentration of soluble nutrients are higher than the outer surface and this makes it difficult to extract microbial DNA from forage sample since there are chances of getting most of the DNA as plant DNA and therefore molecular analysis would provide us with the accurate results of microbial DNA in each sample per gram forage to conclude which extraction protocol would result in the maximum yield [40]. Although, for DNA, dried samples can be ball milled before the extraction but this procedure can increase the amount of residual plant DNA. Thus, this probability of reduction in the ratio of microbial DNA to plant DNA can decrease the sensitivity of analysis too. Hence, liquid based extraction method was used to harvest cell pellets from forage before nucleic acid isolation.

2.3 Sample Preparation

10 g of forage sample was mixed with 0.85% of sterile sodium chloride solution and kept on a shaker at 120 rpm and incubated for 15 minutes [40]. The sample was then centrifuged at 10,000 rpm for 10 minutes at 4 °C and supernatant was discarded. The cell pellets were resuspended in 1 ml of sterile 0.85% NaCl solution and centrifuged again at 10,000 rpm for 15 minutes at 4 °C. The supernatant was discarded and resulting cell pellets were then used to extract microbial DNA.

2.4 Material and Methods

After cell pellets were collected, Qiagen DNeasy power soil kit was used to extract DNA with three different bead beating durations. Each sample was closed in Beadmill 24 Homogenizer (110/220V) from Fisherbrand for bead beating. Three different bead beating durations were selected, 1 minute (one cycle of shaking), 4 minutes, (2 cycles of 2 minutes shaking, with a 30 second pause after each cycle) and 9 minutes (4 cycles of 2 minute and 1 cycle of 1 minute, with a 30 second pause after each cycle. Each sample was placed at a speed of 2200 rpm and was maintained at the room temperature throughout. Following bead beating step, DNA was purified followed by the protocol of Qiagen and 100ul was eluted for downstream applications. The samples were eluted twice to ensure all of the DNA was extracted from the spin column in the first elution. Each set of duration was tested in triplicates. Qiagen Blood and Tissue kit that utilized proteinase K for cell lysis was used as the second commercial kit. Three different incubation durations were selected. Samples in triplicated were incubated at 55 °C for 10 minutes, 20 minutes and 60 minutes for cell lysis. In the final elution step, 200µl of DNA was collected and eluted twice to verify if maximum amount of DNA has been collected from the first elution.

2.5 Results

DNA concentration (ng DNA/µL) and $A_{260/280}$ ratio (absorbance at 260 nm/absorbance 280 nm) of each extract was determined spectrophotometrically using a nanodrop spectrophotometer. The yield for each DNA extraction method was calculated as follows: Yield of extraction (µg of DNA/g of sample) = concentration of DNA in the extract (ng/µl) x (1µg/1000ng) x final volume of extract (µl)/ dry weight of sample(g). In addition to DNA extraction, in the final step of elution, each DNA

sample was incubated for 37 degrees for 15 minutes. A subset of experiments without incubating at the final step was also carried out to compare the effect of incubation at elution.

Table 3 A comparison of Qiagen Power Soil kit and Qiagen Blood and Tissue Kit.

	Time	$A_{260/280}$	DNA Concentration	Average	Yield	Incubation
Qiagen	**minutes**		**ng/μL**	**ng/μL**	**μg/g forage**	
Power Soil Kit	1	1.843	21.965	23.856	0.239	
		1.825	28.992			
		1.938	20.61			
	4	1.831	36.937	29.468	0.295	
		1.833	30.767			
		1.841	20.701			
	9	1.855	30.645	26.081	0.261	
		1.874	20.53			
		1.917	27.067			
Blood and Tissue Kit	10	1.648	19.148	18.994	0.380	
		1.726	15.38			
		1.703	22.454			
		1.934	8.974	13.836	0.277	-
		1.54	16.793			-
		1.416	15.742			-
	20	1.717	20.379	18.725	0.375	
		1.688	18.46			
		1.744	17.336			
		1.922	7.54	22.090	0.442	-
		1.474	50.448			-
		2	8.281			-
	60	1.755	22.066	26.277	0.526	
		1.737	38.434			
		1.668	18.331			
		1.681	9.594	9.306	0.186	-
		1.758	8.804			-
		1.757	9.52			-

2.6 Discussion

The selection of an appropriate method for extracting DNA from forages has a critical impact on understanding the maximum amount of microbial DNA that can be extracted from total (microbial

+ plant) DNA [41]. The performance of eighteen extracted samples using two different commercial kits and four different criteria were evaluated to identify the parameters to obtain PCR quality microbial DNA including three different bead beating time for mechanical disruption and three different incubation duration for enzymatic cell disruption. In the final step each extract was eluted twice to verify the effect of multiple elution on extracted DNA and the effect of incubating before extracting the DNA from spin column for downstream applications. Different bead beating durations did not increase the overall yield of extracted DNA and the quality of DNA was not compromised. Results after incubating with proteinase K were not statistically significant however incubating at the elution step was critical to extract the maximum DNA in the spin column. There is a high probability of the intrusion of plant DNA along with the microbial DNA extracted therefore, it is suggested to compare 16SrRNA copy number to total DNA quantity using real time quantitative polymerase chain reaction (qPCR).

References

[1] M. J. Blaser *et al.*, "Toward a predictive understanding of earth's microbiomes to address 21st century challenges," *MBio*, vol. 7, no. 3, pp. 1–16, 2016.

[2] I. Soundharrajan *et al.*, "Application of customised bacterial inoculants for grass haylage production and its effectiveness on nutrient composition and fermentation quality of haylage," *3 Biotech*, vol. 7, no. 5, pp. 1–9, 2017.

[3] C. E. Müller, D. von Rosen, and P. Udén, "Effect of forage conservation method on

microbial flora and fermentation pattern in forage and in equine colon and faeces," *Livest. Sci.*, vol. 119, no. 1–3, pp. 116–128, 2008.

[4] S. Citation, *Nutrient Requirements of Dairy Cattle*. 2001.

[5] H. J. S. Finch, A. M. Samuel, and G. P. F. Lane, "Conservation of grass and forage crops," *Lockhart Wiseman's Crop Husb. Incl. Grassl.*, pp. 513–526, 2014.

[6] T. F. Bernardes, J. R. S. Gervásio, G. De Morais, and D. R. Casagrande, "Technical note: A comparison of methods to determine pH in silages," *J. Dairy Sci.*, vol. 102, no. 10, pp. 9039–9042, 2019.

[7] S. D. Hafner *et al.*, "Emission of volatile organic compounds from silage: Compounds, sources, and implications," *Atmos. Environ.*, vol. 77, pp. 827–839, 2013.

[8] J. C. Murrell, I. R. McDonald, and D. G. Bourne, "Molecular methods for the study of methanotroph ecology," *FEMS Microbiol. Ecol.*, vol. 27, no. 2, pp. 103–114, 1998.

[9] C. M. Jones, D. R. H. Graf, D. Bru, L. Philippot, and S. Hallin, "The unaccounted yet abundant nitrous oxide-reducing microbial community: A potential nitrous oxide sink," *ISME J.*, vol. 7, no. 2, pp. 417–426, 2013.

[10] E. Pattillo, Gary, "Fast Facts from the Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2017," *Coll. Res. Libr. News*, vol. 80, no. 5, p. 304, 2019.

[11] M. Hayatsu, K. Tago, and M. Saito, "Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification," *Soil Sci. Plant Nutr.*, vol. 54, no. 1, pp. 33–45, 2008.

[12] N. Wrage, G. L. Velthof, M. L. Van Beusichem, and O. Oenema, "Role of nitrifier denitrification in the production of nitrous oxide," *Soil Biol. Biochem.*, vol. 33, no. 12–13, pp. 1723–1732, 2001.

[13] C. A. Francis, K. J. Roberts, J. M. Beman, A. E. Santoro, and B. B. Oakley, “Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 41, pp. 14683–14688, 2005.

[14] D. A. Stahl and J. R. De La Torre, “Physiology and diversity of ammonia-oxidizing archaea,” *Annu. Rev. Microbiol.*, vol. 66, pp. 83–101, 2012.

[15] T. Zhang, T. Jin, and Q. Yan, “Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR,” *Appl. Microbiol. Biotechnol.*, vol. 87, no. 3, pp. 1167–1176, 2010.

[16] S. Henry, E. Baudoin, J. C. López-Gutiérrez, F. Martin-Laurent, A. Brauman, and L. Philippot, “Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR,” *J. Microbiol. Methods*, vol. 59, no. 3, pp. 327–335, 2004.

[17] L. A. Domeignoz-Horta *et al.*, “Non-denitrifying nitrous oxide-reducing bacteria - An effective N2O sink in soil,” *Soil Biol. Biochem.*, vol. 103, pp. 376–379, 2016.

[18] R. A. Sanford *et al.*, “Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 48, pp. 19709–19714, 2012.

[19] A. R. Ravishankara, J. S. Daniel, and R. W. Portmann, “Nitrous oxide (N2O): The dominant ozone-depleting substance emitted in the 21st century,” *Science (80-.).*, vol. 326, no. 5949, pp. 123–125, 2009.

[20] K. I. Kostyanovsky, D. R. Huggins, C. O. Stockle, S. Waldo, and B. Lamb, “Developing a flow through chamber system for automated measurements of soil N2O and CO2 emissions,” *Meas. J. Int. Meas. Confed.*, vol. 113, pp. 172–180, 2018.

[21] G. Flux, “Gas Flux,” vol. 9, no. 9, pp. 1103–1119, 1986.

[22] W. Wei *et al.*, "Development of PCR primers targeting fungal nirK to study fungal denitrification in the environment," *Soil Biol. Biochem.*, vol. 81, pp. 282–286, 2015.

[23] V. Michotey, V. Méjean, and P. Bonin, "Comparison of methods for quantification of cytochrome cd1-denitrifying bacteria in environmental marine samples," *Appl. Environ. Microbiol.*, vol. 66, no. 4, pp. 1564–1571, 2000.

[24] G. Braker, A. Fesefeldt, and K. P. Witzel, "Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples," *Appl. Environ. Microbiol.*, vol. 64, no. 10, pp. 3769–3775, 1998.

[25] W. Wei *et al.*, "Higher diversity and abundance of denitrifying microorganisms in environments than considered previously," *ISME J.*, vol. 9, no. 9, pp. 1954–1965, 2015.

[26] K. Heylen *et al.*, "The incidence of nirS and nirK and their genetic heterogeneity in cultivated denitrifiers," *Environ. Microbiol.*, vol. 8, no. 11, pp. 2012–2021, 2006.

[27] G. Braker and J. M. Tiedje, "Nitric oxide reductase (norB) genes from pure cultures and environmental samples," *Appl. Environ. Microbiol.*, vol. 69, no. 6, pp. 3476–3483, 2003.

[28] H. J. Park, J. H. Kwon, J. Yun, and K. S. Cho, "Characterization of nitrous oxide reduction by Azospira sp. HJ23 isolated from advanced wastewater treatment sludge," *J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng.*, vol. 55, no. 12, pp. 1459–1467, 2020.

[29] K. F. Ettwig, T. Van Alen, K. T. Van De Pas-Schoonen, M. S. M. Jetten, and M. Strous, "Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum," *Appl. Environ. Microbiol.*, vol. 75, no. 11, pp. 3656–3662, 2009.

[30] F. S. Colwell, S. Boyd, M. E. Delwiche, D. W. Reed, T. J. Phelps, and D. T. Newby, "Estimates of biogenic methane production rates in deep marine sediments at Hydrate

Ridge, Cascadia margin," *Appl. Environ. Microbiol.*, vol. 74, no. 11, pp. 3444–3452, 2008.

[31] J. Im, S.-W. Lee, L. Bodrossy, M. J. Barcelona, and J. D. Semrau, "Field application of nitrogen and phenylacetylene to mitigate greenhouse gas emissions from landfill cover soils: effects on microbial community structure," *Appl. Microbiol. Biotechnol.*, vol. 89, no. 1, pp. 189–200, 2011.

[32] C. J. Smith, D. B. Nedwell, L. F. Dong, and A. M. Osborn, "Diversity and abundance of nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nrfA), and their transcripts in estuarine sediments," *Appl. Environ. Microbiol.*, vol. 73, no. 11, pp. 3612–3622, 2007.

[33] G. Angnes *et al.*, "Correlating denitrifying catabolic genes with N2O and N2 emissions from swine slurry composting," *Bioresour. Technol.*, vol. 140, pp. 368–375, 2013.

[34] S. T. Paul, "This Article Is Sponsored By the Minnesota Dairy Health Conference .," *Prevention*.

[35] R. Area, "Impacts of Elevated CO 2 Levels on the Soil Bacterial."

[36] T. R. Callaway *et al.*, "Sodium chlorate supplementation reduces E. coli O157:H7 populations in cattle," *J. Anim. Sci.*, vol. 80, no. 6, pp. 1683–1689, 2002.

[37] O. C. M. Queiroz, K. G. Arriola, J. L. P. Daniel, and A. T. Adesogan, "Effects of 8 chemical and bacterial additives on the quality of corn silage," *J. Dairy Sci.*, vol. 96, no. 9, pp. 5836–5843, 2013.

[38] T. A. McAllister *et al.*, "Silage review: Using molecular approaches to define the microbial ecology of silage," *J. Dairy Sci.*, vol. 101, no. 5, pp. 4060–4074, 2018.

[39] S. Bag *et al.*, "An improved method for high quality metagenomics DNA extraction from

human and environmental samples," *Sci. Rep.*, vol. 6, no. 4, pp. 1–9, 2016.

[40] B. Liu, H. Huan, H. Gu, N. Xu, Q. Shen, and C. Ding, "Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages," *Bioresour. Technol.*, vol. 273, no. 50, pp. 212–219, 2019.

[41] L. Kung, R. D. Shaver, R. J. Grant, and R. J. Schmidt, "Silage review: Interpretation of chemical, microbial, and organoleptic components of silages," *J. Dairy Sci.*, vol. 101, no. 5, pp. 4020–4033, 2018.

[42] K. M. Ritalahti, B. K. Amos, Y. Sung, Q. Wu, S. S. Koenigsberg, and F. E. Löffler, "Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains," *Appl. Environ. Microbiol.*, vol. 72, no. 4, pp. 2765–2774, 2006.

Target	Name	Sequence	Method
Bacterial 16S	8F 1492R	AGAGTTTGATCCTGGCTCAG TACCTTGTTACGACTT	1m@95(45s@94,45s@50,1.5m@72) x26, 15m@72
Bacterial 16S	335F 769R	CAAACTCCTACGGGAGGC ATCCTGTTTGMTMCCCVCRC	10m@95, (10s@95, 10s@59, 90s@72)40x, 10m@72
Bacterial 16S	1055F 1392R	ATGGYTGTCGTCAGCT ACGGGCGGTGTGTAC	10m@95,(30s@95, 1min@50, 20s@72)35x, 10m@72
Archaeal 16S	A571F UA1204R	GCYTAAAGSRICCGTAGC TTMGGGGCATRCIKACCT	2m@94, 30X(60s@94, 60s@55, 60s@72), 10m@72
Archaeal 16S	931f m1100r	AGGAATTGGCGGGGGAGCA BGG GTC TCG CTC GTT RCC	15m@95, 45X(15s@94, 30s@64, 30s@72), 7m@72 , 30s@95, 40X(15s@95, 30s@60, 15s@72) , 2m@95,
Nitrate Reductase	narG1960F narG2650R	TAYGTSGGSCARGARAA TTYTCRTACCABGTBGC	5m@94,(30s@94,30s@55, 1m@72)x35, 10m@72
Nitrite Reductase	nirKC1F nirKC1R	ATGGCGCCATCatggtnytncc TCGAAGGCCTCGatnarrttrtg	10m@95, (30s@95, 30s@54, 30s@72), 10m@72
Nitrite Reductase	nirKC2F nirKC2R	TGCACATCGCCAACggnatgtwygg GGCGCGGAAGATGshrtgrtcnac	10m@95, (30s@95, 30s@54, 30s@72), 10m@72
Nitrite Reductase	cd8F cd2R	GGNTAYGCNGTNCAYAT CCNGTYTCYTTNACRTTNAC	2m@94, (30s@94, 30s@40, 40s@72), 3m@72
Nitric Oxide Reductase	qnorB2F qnorB5R	GGNCAYCARGGNTAYGA ACCCANAGRTGNACNACCCACCA	5m@95, 10x(30s@95, 40s@(touchdown from 57 to 52.5), 60s@72), 30X(30s@95, 40s@55, 60s@72), 10m@72
Nitric Oxide Reductase	cnorB2F cnorB7F	GACAAGNNNTACTGGTGGT TGNCCRTGNGCNGCNGT	5m@95, 10x(30s@95, 40s@(touchdown from 57 to 52.5), 60s@72), 30X(30s@95, 40s@55, 60s@72), 10m@72
Nitrous Oxide Reductase	nosZf nosZr	AACGACAAGDYCAA AKSGCRTGGCAGAA	3m@95, 35X(60s@94, 60s@55, 120s@72), 3m@72
Nitrous Oxide Reductase	nosZ1F noszZ1R	WCSYTGTTCMTCGACAGCCAG ATGTCGATCARCTGVKCRTTYTC	3m@95, 35X(60s@94, 60s@55, 120s@72), 3m@72
Nitrous Oxide Reductase	nosZ2F nosZ2R	CTIGGICCIYTKCAYAC GCIGARCARAAITCBGTRC	3m@95, 35X(60s@94, 60s@55, 120s@72), 3m@72
Bacterial amoA	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	5m@94, (60s@94, 90s@60, 90s@72), 10m@72, x 40cycle

Archaeal amoA	amoAF]amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	5m@95, 30 cycle (45s@94, 60s@53, 60s@72), 15m@72, 45 cycle
pmoA	A189f mb661r	GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	15m@95, (25s@95, 20s@65, 45s@72), 30m@72.
mmoX	mmoX206f mmoxX886r	ATCGCBAARGAATAYGCSCG ACCCANGGCTCGACYTTGAA	5m@95, (60s@94, 90s@60, 90s@72), 10m@72

Table 4 Primer Sequence targeting functional genes