Controlling the Process of Denitrification in Flooded Rice Soils by Using Microbial Fuel Cell Applications

Controlling the Process of Denitrification in Flooded Rice Soils by Using Microbial Fuel Cell Applications

(微生物燃料電池を援用した湛水水田土壌における脱窒過程の制御)

2017

The United Graduate School of Agricultural Science,
Gifu University
Science of Biological Environment

(Gifu University)

Ranatunga Arachchige Tharangika Ranatunga

Controlling the Process of Denitrification in Flooded Rice Soils by Using Microbial Fuel Cell Applications

(微生物燃料電池を援用した湛水水田土壌における脱窒過程の制御)

Ranatunga Arachchige Tharangika Ranatunga

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Notations

MFCs	-	microbial fuel cells
Non-MFCs	-	non-microbial fuel cells
MFC-extV	-	microbial fuel cells plus external voltage applied
sMFC	-	sediment microbial fuel cell
NH_4^+	-	ammonium
NO_3^-	-	nitrate
NO_2^-	-	nitrite
NO	-	nitric oxide
N_2O	-	nitrous oxide
N_2	-	di-nitrogen
N	-	Nitrogen
C	-	Carbon
O_2	-	oxygen
CO_2	-	carbon dioxide
CH_4	-	methane
Mn^{4+}	-	manganese
Fe^{3+}	-	Ferric
SO_4^{2-}	-	sulfate
TN	-	total nitrogen
TC	-	total carbon
$C_6H_{12}O_6$	-	glucose
H_2O	-	water
Eh	-	redox potential
DNRA	-	dissimilatory nitrogen reduction to ammonia
PCR	-	polymerase chain reaction
ECD	-	electron capture detector
v/v %	-	volume to volume percentage
m/v	-	mass per volume
$(NH_4)_2SO_4$	-	ammonium sulfate
ppm	-	parts per million
ppb	-	parts per billion
GWP	-	global warming potential
GHG	-	greenhouse gas

ACKNOWLEDGMENTS

First of all, I'd be very much grateful to Professor Ken Hiramatsu of Faculty of Applied Biological Sciences of Gifu University for introducing me to the challenging topic, and for the invaluable guidance and support extended there as my immediate supervisor. It is with great honour that I convey my deepest gratitude for your understanding and encouragement throughout these years.

My sincere thanks and gratitude goes to Professor Takeo Onishi of Faculty of Applied Biological Sciences of Gifu University, the co-supervisor of my research and for all your invaluable guidance and support extended throughout my research.

My sincere thanks and gratitude goes to Professor Yasushi Ishiguro of River Basin Research Center of Gifu University for his invaluable guidance and support extended throughout my research.

Next, I would like to extend my sincere thanks to Professor Imaizumi of Shizuoka University for his support during the online sessions of my research presentations between the two universities.

In addition, I'm thankful to Professor Toshiaki Iida of The University of Tokyo and Professor Fusheng Li of River Basin Research Center, Gifu University for their advice on our research work.

My sincere thanks goes to the staff of The United Graduate School of Agricultural Science for providing me the support throughout the course. Next, I'm very much grateful for the Program of Basin Water Environmental Leaders (BWEL), Gifu University, and the staff of River Basin Research Center. Especially I'd like to thank Professor Yongfeng Wei, the co-ordinator of the BWEL program, for accepting and supporting me towards the success of BWEL program at Gifu University. At the same time, I'd like to appreciate for each and every moment that I spent with my colleagues and my juniors.

I'm very much grateful to you, Mr. Hisao Hayashi, the former English teacher of Nagoya Municipal Koyo Senior High School, and who has been then continued teaching as a part-time lecturer at Nagoya University of Foreign Studies. It was a great chance to hear about Gifu University to the first time from Mr. Hisao Hayashi that inspired me to think about studying there.

All in all, I'd like to appreciate my family for their continuous support and encouragement extended throughout the time.

Also, it is noteworthy that the work was supported in part by JSPS KAKENHI Grant number 15H04567 and by the New Energy and Industrial Technology Development Organization (NEDO) under the Ministry of Economy, Trade and Industry of Japan.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

1.1 Problem of Nitrogen fertilizer Deficiency

The global food industry is highly dependent on fossil fuels. Energy-efficient approaches to agriculture would offer a way to take advantage of the relationships between energy, food, and agriculture. A huge amount of energy is required for the fixation of nitrogen fertilizer from the unlimited atmospheric nitrogen. To manufacture one metric ton of anhydrous ammonia, NH_3 (82% N), it is estimated that 3,500 m^3 of natural gas is used (Olsan and Halstead, 1974). It is also estimated that the field application of 150 kg/ha nitrogen fertilizer in the form of ammonia involves the consumption of 645 cubic meters of natural gas (Olsan and Halstead, 1974). Moreover, despite the fact that rice is the staple food of half the world's population, nitrogen fertilizer is not used efficiently (Keeny et al., 1986). Recognizing the fact that nitrogen fertilizer is absolutely essential for supporting the growing global population, there is an urgent need to explore efficient ways of using fertilizer to compensate for future food shortages. Although various methods such as leguminous crop production, crop rotation, and management of irrigation water have been proposed and discussed (Huang et al., 2007; Kaewpradit et al., 2008; Pramanik et al., 2014), these are indirect methods. Improving N retention efficiencies in crop soils would be one of the challenges to date. To improve fertilizer efficiency, a more direct way of controlling the nitrogen transformation processes in the soil would be more promising. Therefore,

1.2 Denitrification redox reaction

The process of denitrification, involving the conversion of soil inorganic nitrogen to elemental nitrogen gas, is one of the main routes behind nitrogen deficiency in crop production. Here, 'denitrification' refers to the process in which NO_3^- is converted to gaseous compounds such as NO, N_2O, and N_2 by microorganisms, and is discussed further in the chapter of Literature review. In submerged soils, the denitrifying bacteria use NO_3^- in the absence of oxygen as the terminal electron acceptor in their process of respiration, which is reduced to NO_2^-, NO, N_2O, and finally N_2 (Reddy and Patrick, 1986). This is considered undesirable for agriculture due to the loss of applied nitrogen fertilizer in soil. In addition, paddy fields have been recognized as one of the sources of atmospheric N_2O (Mosier

et al., 1996; Akiyama et al., 2005), one of the greenhouse gases causing global warming. Thus, the extent of nitrogen loss via denitrification has attracted great interest among researchers as well as farmers (Iida et al., 2007).

1.3 Objective of the study and Approach to the Solution

Main objective of this research is to investigate the applicability of MFC to control soil redox potential and thereby to suppress denitrification, namely, nitrogen losses in flooded rice fields.

1.3.1 Redox potential

Redox, termed short for oxidation-reduction, denotes the transfer of electrons between two species. Reduction is the gain of electrons by a compound, whereas oxidation is the loss of electrons by a compound. The electron donors are usually reduced carbon and the acceptors are usually oxygen in aerobic environments. Oxidation-reduction conditions are classically assessed by measuring the redox potential (Eh), expressed in volts. It is a measure of electron activity in a system. Microorganisms are probably the most important factor controlling the Eh of soils: as they reduce carbon to store energy, they oxidize it to release energy and they use oxygen or other molecules as electron acceptors, thus forming reduced species under oxygen limiting conditions (Lambers *et al*., 2013). O_2 has the highest redox potential, followed by other alternative electron acceptors: $NO_3^- > Mn^{4+} > Fe^{3+} > SO_4^{2-}$.

However, redox potential (Eh) has received little attention in agronomy, unlike pH, which is regarded as a master variable (Husson *et al.*, 2013). This would be due to the fact that Eh measurements are difficult to reproduce and interpret according to Snakin *et al*., (2001). In many disciplines, oxidation-reduction conditions and electron fluxes have not received the same attention as have pH and proton fluxes (Husson *et al.*, 2013). The control of redox process is a proper niche for the unique capabilities of a bio-electrochemical system (Arends and Verstraete, 2012), since microbes participate in biochemical reactions to satisfy their energy needs and resource demands.

1.3.2 Microbial Fuel Cell (MFC) concept

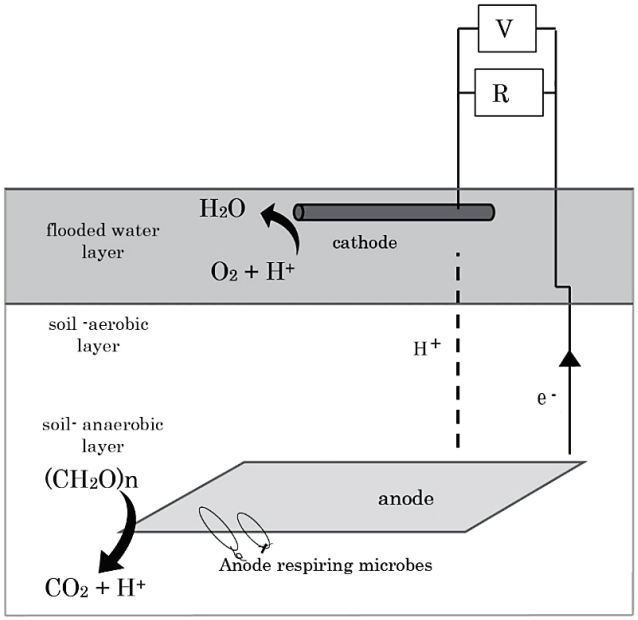


Figure 1.1 Scheme of a microbial fuel cell: A carbon graphite-felt mat is used as anode placed in the anaerobic soil layer, and a graphite rod is kept floating on the flooded water in contact with air. The anode and cathode are linked through insulated wires externally connected with a resister (R) and a voltmeter (V).

According to the definition given by Lovley (2006), a microbial fuel cell (MFC) is a device that converts chemical energy into electrical energy. Generating power in MFCs depends on redox chemistry. Microbes can gain energy through redox reactions between organic compounds and terminal electron acceptors such as O_2. This process can be interpreted as involving the oxidation of the organic compound and the reduction of the terminal electron acceptor. The principle of MFCs involves the utilization of electrons liberated by the decomposition of organic matter by microbes to produce electricity. In other words, MFCs are devices that exploit microorganisms to generate electric power from organic matter.

The concept of an MFC can also be applied to flooded rice soils, which can then be referred to a paddy field microbial fuel cell. **Figure 1.1** illustrates the concept of an MFC applied to flooded soils. The electrons produced during the oxidation of organic matter are transferred directly to an anode, and the electrons travel through an electrical circuit to a cathode where oxygen is reduced to H_2O. While the region several centimeters below the flooded soil surface is anaerobic, near the soil surface, aerobic

conditions prevail. Thus, potential gradients could be generated between these regions, when connected externally through insulated wires. It is therefore possible to generate electricity if anode respiring microbes have self-sustained extracellular electron transfer mechanisms (Lovley, 2008). In fact, some studies have already shown that electricity can be produced in flooded soils (Arends et al., 2014; Kaku et al., 2008; Kouzuma et al., 2014; Takenezawa et al., 2010).

In this context, the following question arises: How can the denitrification process be related to the principle of MFCs? In the denitrification process of flooded rice fields, the electrons produced from the oxidation of organic matter by microbes are gained by oxygen-containing inorganic nitrogen species such as NO_3^- and NO_2^-. Typical redox reactions related to NO_3^- respiration that are performed by microbes in the absence of oxygen are shown in the following equations.

$$5\,C_6H_{12}O_6 + 24\,NO_3^- + 24\,H^+ \rightarrow 30\,CO_2 + 12\,N_2 + 42\,H_2O \quad (1)$$

$$C_{12}H_{22}O_{11} + 9.6\,NO_3^- + 9.6\,H^+ \rightarrow 12\,CO_2 + 4.8\,N_2 + 15.8\,H_2O \quad (2)$$

Because these are redox reactions between organic carbon and oxidized forms of nitrogen anions, the availability of organic carbon should be one of the factors determining whether denitrification occurs. The dynamics of organic carbon is difficult to control, whereas in contrast the electrons resulting from its oxidation through microbial metabolism can be controlled. Specifically, the electrons resulting from the oxidation of organic matter can be circulated in a chain, which is referred to as an electric chain, when the anaerobic layer is externally connected to the upper aerobic layer of soils. MFC could be a driver of change of electron activity in the system or redox potential changes. It is therefore hypothesized that denitrifying conditions could be controlled by redox potential changes in soils that are invoked by MFC. In other words, a competition for electrons is expected to invoke by the generation of electricity, and to outcompete denitrification reactions at the same time. Thus, in this study, we investigated the applicability of MFC to control soil redox potential and thereby to suppress denitrification, namely, nitrogen losses in flooded rice fields.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

2.1.1 Nitrogen: An element essential for life

Nitrogen is a fundamental component of living organisms that can be assimilated by plants in both marine and land ecosystems. As a result, nitrogen has a critical role in controlling primary production in the biosphere (Gruber and Galloway, 2008). Understanding nitrogen transformations and the soil microbes that perform the transformations are thus essential for managing ecosystem health and productivity (Groffman *et al*., 2015). Despite nitrogen being one of the most abundant elements on earth, nitrogen deficiency is probably the most common nutritional problem affecting plants. Nitrogen is an essential nutrient for plant growth. Soil nitrogen are in three general forms as organic nitrogen compounds, ammonium (NH_4^+) ions and nitrate (NO_3^-) ions. Although, the most available nitrogen in the soils is in organic forms, organic nitrogen is not directly available to plants, but some can be converted to available forms by microorganisms. The majority of plant-available nitrogen is in the inorganic forms as NH_4^+ and NO_3^- which are called "mineral nitrogen".

2.1.2 Process of denitrification in Nitrogen cycle

Denitrification is the only point in the N cycle where fixed N re-enters the atmosphere as N_2; it thus serves to close the global N cycle. Denitrification is also significant as the major source of atmospheric N_2O, an important greenhouse gas that also consumes stratospheric ozone (Groffman *et al*., 2015). Denitrification refers to the process in which nitrogen oxides are converted to gaseous compounds (nitrate, nitrite, nitric oxide, nitrous oxide and N_2) by microorganisms (Wrage *et al*., 2001). Denitrification is accelerated under anaerobic (flooded or compacted) conditions and with high nitrogen inputs. The conditions favourable are a supply of oxidizable organic matter, absence of oxygen and availability of reducible nitrogen sources. Several types of bacteria perform this conversion when growing on organic matter in anaerobic conditions. Mostly heterotrophic and facultative anaerobes are able to use NO_3^- in place of O_2 in respiration. It is a stepwise reduction of NO_3^- to N_2 where N_2O is a regular intermediate (Wrage *et al*., 2001), as shown in **Figure 2.1.**

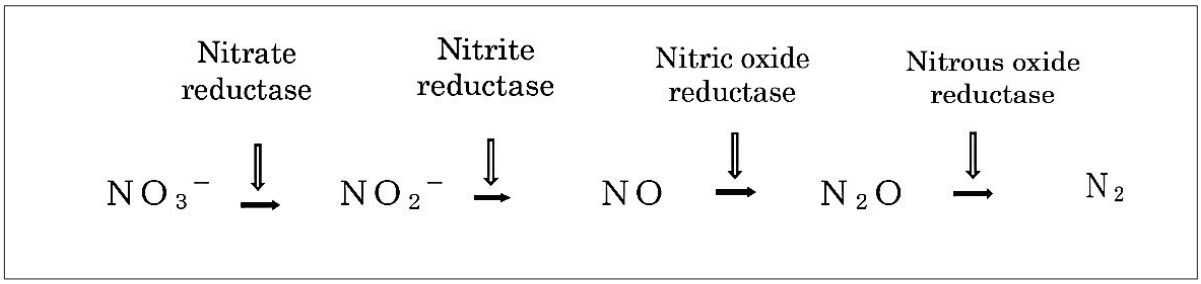


Figure 2.1 Stepwise reduction of NO_3^- to N_2 where N_2O is a regular intermediate (Adapted from Wrage et al., 2001)

In the absence of oxygen, any reducible substance such as nitrate (NO_3^-) could serve the same role and be reduced to nitrite, nitric oxide, nitrous oxide or N_2, as the microbes use NO_3^- instead of O_2 as a terminal electron acceptor. This is also termed as anaerobic respiration which involves a redox reaction between organic carbon and oxidized forms of nitrogen anions as shown in the equation (1) and (2) in chapter1.

The electron donor can be any biodegradable carbon compound, but easily degradable, low molecular weight compounds are preferred; such as acetate or methanol. According to Beauchamp *et al*. (1989), availability of organic carbon is one of the most important factors that affect denitrifying activity in soil, yet the chemistry of soil organic matter is only partially understood and its complexity has probably inhibited studies. Therefore, a readily available carbon substrate is added to ensure an electron supply in the denitrifying system, when other variables are studied in a laboratory (Beauchamp *et al*., 1989). The rates of denitrification in soil are controlled by numerous biological, chemical, and physical factors that are constantly changing both in time and space. The level of organic matter, oxygen concentration, gas diffusion rates, and pathways of diffusion, moisture, temperature, pH and substrate availability (nitrate, nitrite) are several factors. In most soils, this nitrogen transformation mainly occurs following rainfall as soil pores become water saturated, and the diffusion of O_2 to microsites is drastically slowed. Typically denitrification starts to occur at water-filled pore space concentrations of 60% and higher (Groffman *et al*., 2015). As most denitrifying bacteria are heterotrophs, O_2 concentrations and carbon availability are extremely important parameters that influence microbial denitrification. Currently recognized denitrifying bacteria such as *Hyphomicrobium*, *Paracoccus*,

Pseudomonas and *Thiobacillus* form a very diverse group. The complexity of substrate carbon supply in the soil is enhanced by the range of microorganisms that are capable of denitrification in soil.

2.1.3 Methods assessing denitrification activity

2.1.3.1 Direct measurement

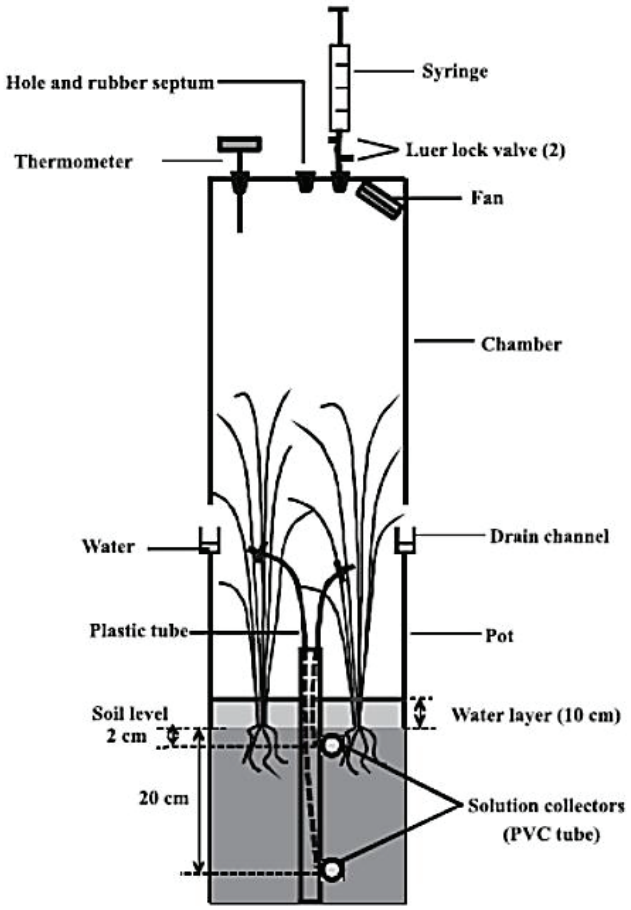


Figure 2.2 Closed chamber for air sampling & soil solution collectors

(Adapted from Tiago et al, 2011)

Determination of denitrification N losses in aqueous and terrestrial systems involves direct gas emission quantifications by gas chromatography using acetylene-based methods (Tiedje *et al*., 1989). C_2H_2 is a specific inhibitor of this denitrification reaction. Acetylene can inhibit the nitrite reductase enzymes of denitrifying microbial which cause N_2O for further reduction to di-nitrogen gas under an atmosphere of 0.1 to 10% (v/v) acetylene. The acetylene inhibition method has been applied in-situ and in field controlled measurements using closed chambers (Ishii *et al*., 2009). Closed chambers used in gas sampling and measurements by Tiago *et al*., (2011) is shown by **figure 2.2**.

2.1.3.2 Mass balance method & 15N tracers

Mass balance method is based on the measure and evolution of NO_3^-, NO_2^- and NH_4^+ concentrations in the liquid phase. Denitrification measures usually imply the addition of organic matter, nitrate as an alternative electron accepter and anaerobic conditions. Isotope - N labelling are mostly limited by the high cost of the isotopes and possible impacts of addition on the environment they are restricted to laboratory for environmental safety reasons. Using these methods involves adding ^{15}N labelled compounds in the form of NO_3^-, NH_4^+ or fertilizers and the subsequent quantification by mass spectrometry of the ^{15}N labelled generated gas compounds.

2.1.3.3 Molecular approaches and functional markers

Denitrification is an anaerobic respiration pathway catalysed by taxonomically diverse bacteria and archaea (Henry et al, 2004). Since the ability of denitrification is distributed among taxonomically diverse groups of bacteria, as well as some archaea and fungi, it is difficult to identify denitrifying organisms based only on their 16s rRNA genes sequence (Philliot et al, 2007). Thus, molecular studies are often directed to the use of functional genes (Henry et al, 2006). Pathway of denitrification reaction and corresponding reductase genes are shown by **figure 2.3**. Molecular analysis are often directed to the functional gene markers of denitrifying bacteria nitrate reductases (nar), nitrite reductases (nir), nitric oxide reductases (nos), and nitrous oxide reductases (nor) reductases. However, primer design seems to be the most critical aspect of microbial environmental diversity surveys (Throback et al., 2004). DGGE of nirK and nosZ genes proved to be a good tool for screening and comparing denitrifying communities in different types of environmental samples, but for nirS gene fragments the resolution was insufficient (Throback et al., 2004).

However, the microbes responsible for denitrifying activity in rice paddy soil are not well known. Denitrification process in bacteria consists of four steps for the reduction of nitrate ions to di-nitrogen ions, catalysed by nitrate, nitrite, nitric oxide, and nitrous oxide reductases. Nitrate reductases are structurally similar among denitrifying bacteria, but are distinct from the enzymes in other nitrate-reducing organisms. They are of two types; as membrane bound nitrate reductases (NarG) and periplasmic nitrate reductases (NapA). The commonly used markers for nitrate reduction include either

membrane-bound (Nar type) or soluble periplasmic (Nap) nitrate reductases, encoded by narG and napA genes, respectively (Henry et al, 2004; Houghton et al, 2001).

Figure 2.3 Pathway of denitrification reaction and corresponding reductase genes

Nitrite reductases (Nir) exist as two functionally equivalent enzymes: a cytochrome cd1-type and a copper containing encoded by the nirS and nirK genes, respectively, which have been thoroughly used as molecular markers for denitrification (Kandeler et al, 2006). Since the nitrate reductase is not always linked to complete denitrification, but may be related to dissimilatory nitrate reduction to ammonia (Philippot et al, 2005), broad range and specific sets for real-time quantification of denitrifiers have been developed based on the nitrite reductase genes nirS and nirK (Gruntzig et al, 2001; Henry et al, 2004 & Throback et al, 2004) and most recently, the nitrous oxide reductase gene nosZ (Henry et al, 2006). The reduction of nitrous oxide that occurs during the last step in the denitrification pathway has received most of the attention in molecular studies. Nitrous oxide reductase mediates the production of nitrogen gas and is coded by the nosZ gene. Nitric oxide reductase has two forms in bacteria, cNOR and qNOR (Zumft et al, 2005). Cytochrome c electron donor is named as cNOR, and quinol electron donor is named as qNOR. cNOR is the most recently associated with denitrifier populations, with qNOR found in some denitrifiers, but also in non-denitrifying microorganisms with a detoxification function against NO (Braker et al, 2003). Nitric oxide reductases are responsible for the conversion of NO to N_2O (Zumft et al, 2005).

2.2 Nitrogen transformations in flooded rice soils

2.2.1 Denitrification: as a major channel of N loss in rice soils

Denitrification in flooded rice soils is affected primarily by oxygen, nitrate and organic carbon availability, whereas nitrification is controlled by oxygen and ammonium availability (Groffman *et al*., 2015). Since submerged rice soil has limited available oxygen, various anaerobic biochemical processes

can occur including methane production, Mn^{4+} and Fe^{3+} reduction, nitrate respiration and denitrification (Ishii *et al*., 2009).

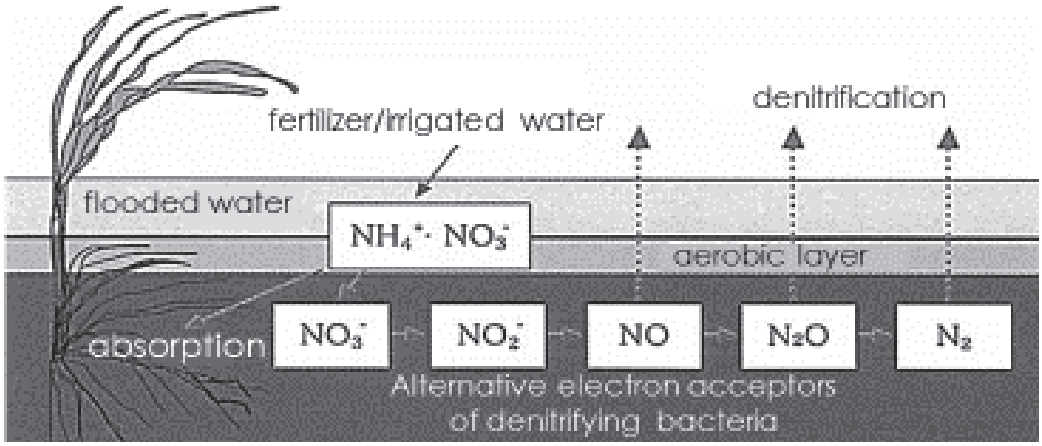


Figure 2.4 Denitrifying activity in flooded rice soils

Rice paddy soil has been shown to have strong denitrifying activity (**figure 2.4**). However, the microbial populations responsible for nitrate respiration and denitrification have not been well characterized (Ishii *et al*., 2009). Flooded rice soil is a dynamic heterogeneous soil-water system that has three distinct soil layers established mainly by the prevailing oxidation-reduction or redox potential of the system (Keeny *et al*., 1986). They consist of partially aerated surface layer (few mm to one cm thickness of surface soil) in contact with flooded water layer, reduced soil and soil rhizosphere. The presence of oxidized zone in close proximity to the reduced zone underlying, is also conductive for the loss of nitrogen through nitrification in oxidized zone followed by denitrification in flooded zone. Ponnamperuma (1972) described the nitrate ion conversion to di-nitrogen gas, indicating that in flooded rice soil, nitrate is extremely unstable, where pE may range from -1 to 3.

Anaerobic layers in wetlands make nitrate in the soil susceptible to gaseous loss to the atmosphere through denitrification to nitrous oxide (N_2O) and dinitrogen (N_2) **(**Hou *et al.,* 2000**)**. N_2O is known as a strong greenhouse gas. Moreover, concern about depletion of stratospheric ozone by oxides of nitrogen has caused considerable interest in the sources of atmospheric N_2O (Crutzen, 1970; Randeniya *et al*., 2002). Rice paddy fields have been recognized as one of the sources of atmospheric N_2O (Minami, 1987; Mosier *et al*., 1996**)**. According to Ghosh *et al*. (2003), the total seasonal N_2O emission from an

irrigated paddy field corresponded to the N loss of 0.10 - 0.12% of applied N. Tropical agricultural soil is attracting significant interest as a source of N_2O, since it is potentially a large emitter of N_2O.

Soil nitrate content and soil water content are the key factors affecting denitrification and N_2O emissions from agricultural soils. Once the soil is flooded, organic matter starts decomposing and is accompanied by a stepwise biochemical reduction of the soil, which is indicated by a lowering of redox potential. Under anaerobic conditions, soil clay which is composed of high carbon content and high nitrogen content, attained negative Eh values within two weeks period after submergence and more negative thereafter and also soil with less carbon took longer to attain similar Eh values (Xiong *et al*., 2006). The reduction of various oxidants in homogeneous soil suspensions occurs sequentially at corresponding soil redox potential (Ponnamperuma, 1972). However, since the N_2O emission from flooded conditions are considered minimal recently, inevitable route of nitrogen movement from paddy soil to the atmosphere should be further explored for the better understanding of nitrogen dynamics in paddy soils (Iida *et al*., 2007). According to Iida *et al* (2007), N_2O emission rate was affected by nitrate-N concentration in the soil water as well as soil temperature, yielding the higher N_2O emission with higher nitrate - N concentration and soil temperature.

2.2.2 Nitrification

Nitrification is the microbial oxidation of ammonia to less reduced forms, principally NO_2^- and NO_3^- (Groffman *et al*., 2015). Nitrification is of two types, as autotrophic and heterotrophic. Autotrophic nitrification is of two types; first ammonia oxidization to nitrite is occurred, consequently with nitrite oxidization to nitrate, as shown in **Figure 2.5**.

Ammonia oxidization is carried out by primary nitrifiers called Nitrosomonas; and then nitrite oxidization is followed by secondary nitrifiers called Nitrobacter. Energy for CO_2 fixation originates from nitrification (Wrage *et al*., 2001). Heterotrophic nitrification involves the same products, intermediates and substrates, but the enzymes are different, while the source of energy is organic carbon. Nitrification is strictly an aerobic microbial process. Lack of oxygen generally inhibits nitrification and greatly influence the rate of ammonification (Keeny *et al*., 1986).

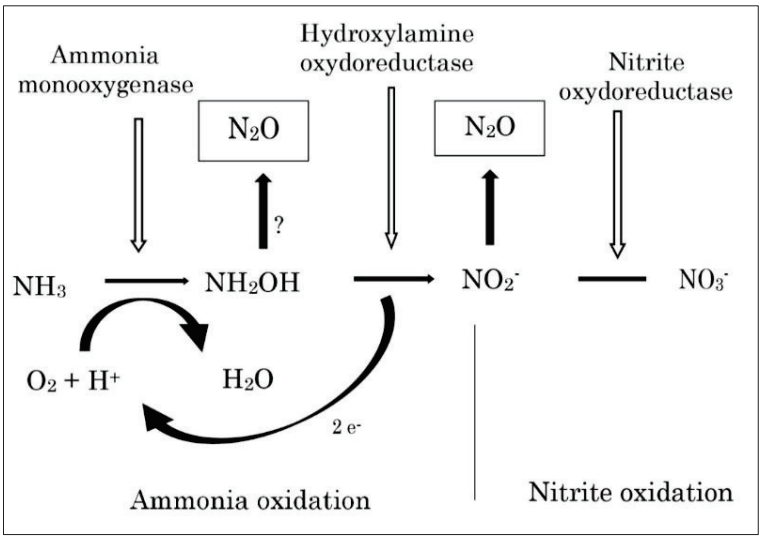


Figure 2.5 Pathways of autotrophic nitrification (Adapted from Wrage et al., 2001)

It is difficult to study nitrification in-situ in a flooded soil system. It is because as soon as NO_3^- is formed, it diffuses down to the reduced layer and is lost by denitrification or is reduced to NH_4^+ by dissimilatory nitrate reduction. However, simultaneous nitrification-denitrification is recognized as a mechanism of N loss in flooded rice fields, of which NO_3^- is an inefficient source of nitrogen therefore. According to Keeny *et al*. (1986), placement of N fertilizer deep in the reduced zone of flooded soil could help in decreasing nitrification. Also by applying the fertilizer when the rice root system established and is being rapidly taken up by plants can help reducing the availability of NH_4^+ for nitrification. As a positively charged ion, ammonium can be held on cation-exchange sites associated with soil organic matter, clay surfaces, and variable-charge minerals. Nitrate, on the other hand, is mostly free in the soil solution and can be easily transported out of the rooting zone by water when precipitation exceeds evapotranspiration (Groffman *et al*., 2015).

2.2.3 Nitrifier denitrification

There is now renewed interest in N balance studies in nitrifier - denitrification as this might be a possible source of N_2O. Role of nitrifier-denitrification in the production of nitrous oxide was reviewed by Wrage *et al*. (2001). The schematic illustration in **Figure 2.6** explains the pathway of nitrifier-denitrification accordingly. This is a nitrification pathway, carried out only by nitrifiers. Oxidation of NH_3 to NO_2^- is followed by a reduction of NO_2^- to N_2O and N_2. This is performed at first by autotrophic

ammonia oxidizers, under the conditions of high O_2 pressure, high Nitrogen, low organic carbon, and low pH. It does not produce an intermediate product of NO_3^- in the pathway of nitrifier-denitrification. Both denitrification and nitrifier - denitrification processes can also overlap.

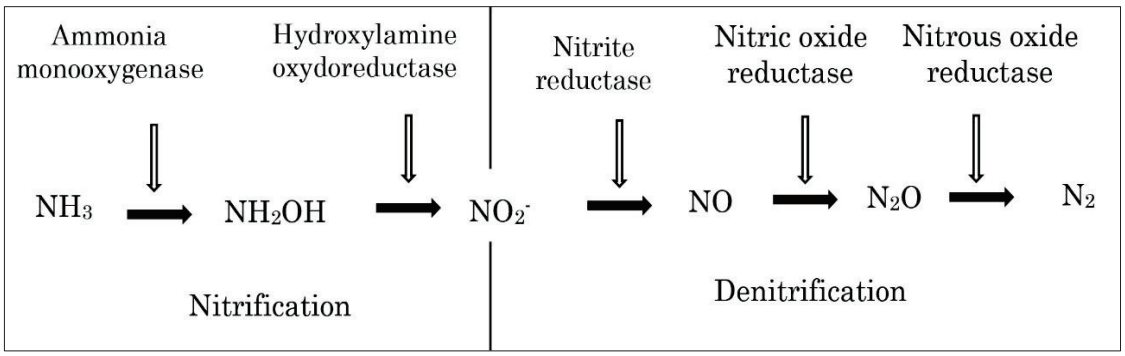


Figure 2.6 Pathway of nitrifier-denitrification (Adapted from Wrage et al., 2001)

2.2.4 Coupled nitrification-denitrification

This can be confused with nitrifier-denitrification. However, NO_3^- can be an intermediate product in the coupled nitrification and denitrification process. NO_3^- is produced in nitrification, and subsequently it can lead to reduction (Wrage *et al*., 2001). NO_2^- or NO_3^- produced by nitrification can then be utilized by denitrifiers. This pathway is mostly possible in microhabitats with both aerobic-(nitrifying) conditions and anaerobic-(denitrifying) conditions.

2.2.5 Volatilization:

Volatilization of nitrogen as ammonia from flooded soils also cause N loss. pH is an important determinant factor of this process. CO_2 concentration and HCO_3^- activity are related with pH fluctuations as shown by Ponnamperuma (1972) according to the equation (2):

$$pH = 7.85 + \log(HCO_3^-) - pCO_2 \quad (2)$$

Therefore, these fluctuations can be responsive to photosynthetic activity and temperature of the system. Increased pH can lead to increased volatilization of ammonia from its surface applied fertilizer or from its diffused NH_4^+ in the water layer. Process of urea hydrolysis can be slowing by using urease inhibitors. NH_3 volatilization could help decreased by using controlled release urea-based fertilizers according to Ponnamperuma (1972).

2.2.6 Mineralization and immobilization

These processes govern the N availability for rice plants which can be occurred simultaneously in flooded soils influenced by soil factors such as pH, organic matter content, C/N ratio, amount and quality of organic material residue and environmental factors such as temperature, soil moisture, regime and soil drying (Keeny *et al*., 1986). Drying of soils enhances N mineralization rate. Mineralization of organic N to NH_4^+ is said to be the key process in the N nutrition of low land rice. Immobilization is also a temperature dependent microbial process, which also increases with temperature. It is a key process in N turnover in lowland rice soils where organic manure is used as N source and is released later to the soil. The balance between N mineralization and immobilization is controlled primarily by organic matter quality, or the availability of carbon in the material relative to its available nitrogen. When one adds to soil manure with a relatively low C: N ratio, the microbes have no trouble obtaining N, and as a result, mineralization dominates over immobilization, and plant-available N increases in soil (Groffman *et al*., 2015).

2.2.7 Diffusion and leaching

Diffusion of NH_4^+ can occur first with the applications of fertilizer. NO_2^- and NO_3^- formed through nitrification in the aerobic flooded layers are diffused downward. NH_4^+ formed through dissimilatory nitrate reduction process can also be diffused. In wetlands and lowland rice soil, diffusion may be restricted most of the time. Leaching may be prevalent in light-textured sandy like soils that are hard to maintain in flooded state. In flooded soils with sandy texture, the losses of N due to leaching could be significant (Keeny *et al*., 1986). Examples of this soils found in Panjab, India with high percolation rates that result in large loss in deep- placed nitrogen. Low CEC also can be a reason. Puddling of soil and compaction are also causative.

2.2.8 Dissimilatory nitrate reduction to ammonia (DNRA)

Another process of denitrification is dissimilatory nitrate reduction to ammonia as shown by equation (3). Basically, DNRA is favoured when nitrate is limiting; whereas denitrification dominates when supply of carbon is limiting. This step blocks the final reduction to nitrogen. Thermodynamically, under

conditions of abundant organic substrate and limited availability of electron acceptors, the reduction of NO_3^- to NH_4^+ is more efficient than forming N_2.

$$NO_3^- + 4\ H_2 + 2\ H^+ \Rightarrow NH_4^+ + 3\ H_2O \quad (3)$$

2.2.9 Anammox

Anammox is a newly discovered microbial way for converting ammonia to nitrogen gas. Anaerobic ammonia oxidizing to nitrogen coupled to use NO_2^- as an electron acceptor. It is believed to account for a significant portion of denitrification occurs world-wide through this process. Availability of nitrate and/or nitrite as electron acceptor is considered an important factor controlling anammox. Part of the process is anaerobic. One half of the ammonia is first oxidized to nitrite aerobically, as shown by equation (4);

$$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + H_2O + 2\ H^+ \quad (4)$$

Next, the nitrite reacts with the remaining ammonia, anaerobically, as shown by equation (5);

$$NH_4^+ + NO_2^- \Rightarrow N_2 + 2\ H_2O \quad (5)$$

2.2.10 Effects of root rhizosphere on N transformations

The plant usable N is either in the form of nitrate (NO_3^-) or ammonium (NH_4^+). Aquatic soils and sediments are characterized by the accumulation of NH_4^+ (Ponnamperuma, 1972). Soil rhizosphere is partially oxidized due to entry of O_2 to rice roots through the rice aerial parts. It may support aerobic nitrogen reactions such as nitrification, mineralization of organic nitrogen and biological N_2 fixation (Keeny *et al*., 1986). Ammonium in the anaerobic zone of the sediment diffuses either into the root zone or into the overlying water column as a result of concentration gradients. Some of the NH_4^+ is either taken up or is oxidized to NO_3^-. The NO_3^- formed is either taken up by the plant or diffused into adjacent anaerobic zones where it is denitrified. To test this hypothesis, Reddy *et al*. (1989) have conducted a series of experiments, with rice (Oryza sativa L.) as a model aquatic plant. In that study, they reported N loss during an experiment designed to maximize nitrification-denitrification reaction in the root zone of rice. The nitrogen losses were measured with the mass balance of added N in the rhizosphere (and with soil column without rice plant) soil systems and it concluded that 29% of NH_4^+ - N was lost through

nitrification-denitrification in 18 days after application in the root zone. Nitrification-denitrification processes in the root zone of rice and other aquatic macrophites as illustrated by Reddy *et al*. (1989) is shown in **Figure 2.7**. However, above nitrogen transformations can be overlapped or existed together as described by **Figure 2.8**.

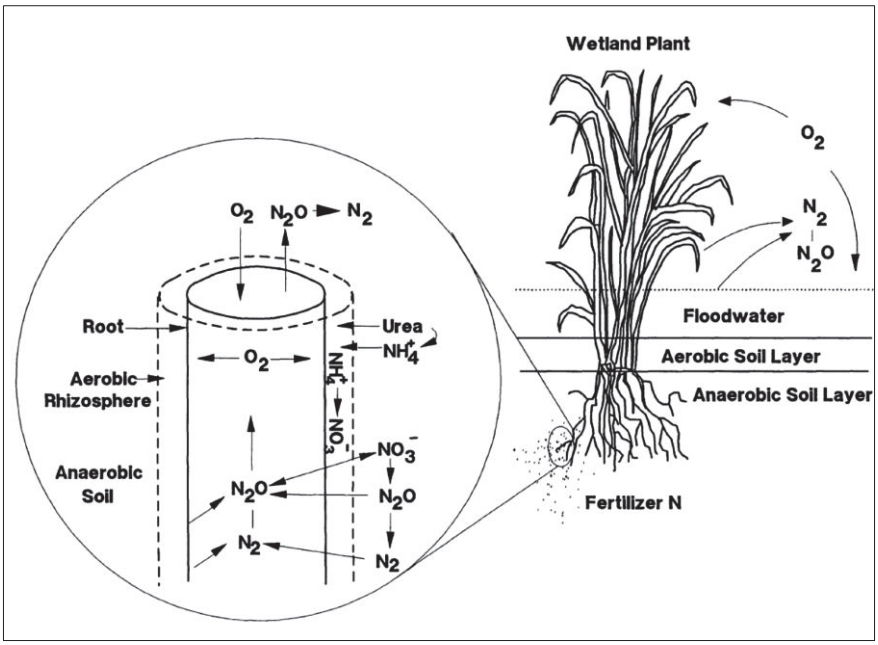


Figure 2.7 Schematic presentation of nitrification-denitrification in the root zone of rice and other aquatic macrophites (adapted from Reddy et al., 1989)

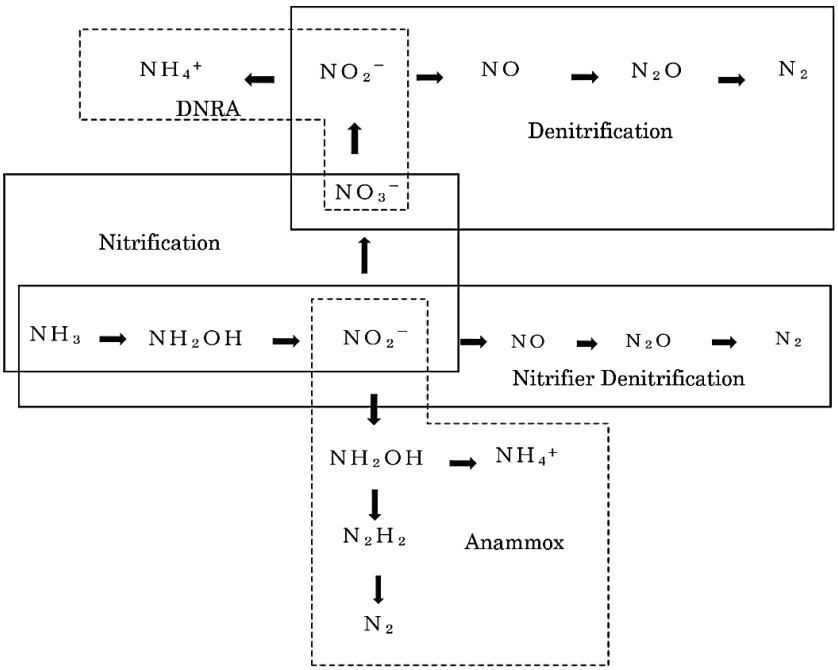


Figure 2.8 Transformations of mineral nitrogen in soil

2.3 GHG emissions in flooded rice systems and their mitigation strategies

As a consequence of denitrification process, Nitrous oxide (N_2O) gas is emitted. It is a potent greenhouse gas that also depletes stratospheric ozone. Rice paddies are regarded as one of the main agricultural sources of N_2O and the available data was summarized by Akiyama *et al*. (2005). To date, however, specific N_2O and NO product pathways are poorly understood in paddy soils. Production of N_2O via denitrification is affected by relative availabilities of electron donors (organic carbon) and electron acceptors (N-oxides), since they are redox reactions.

According to Lan *et al*. (2014), both N_2O and NO emission rates in alkaline paddy soil with clayey texture were substantially higher than those in a neutral paddy soil with silty loamy texture. In accordance with most published data, the ammonium N pool was the main source of N_2O emission across the two soil profiles mentioned by Lan *et al*. (2014). They showed that NO fluxes were positively correlated with the changes in gross nitrification rates and the ratio of NO: N_2O in both paddy soils were always greater than one. Also Lan *et al*. (2014) deduced that, when the water holding capacity is below 60%, nitrification was also dominant source of NO in the paddy soils similar to that of N_2O.
Water regime is one of the key factors affecting nitrous oxide emissions from soils. Huang *et al*. (2007) conducted soil column experiments with three irrigation regimes, namely, flooding, intermittent and natural drying, to gain insights into how irrigation activities on paddy soil in south eastern China affect the dynamic process of N_2O emissions. Accordingly, natural drying had the most contribution to N_2O emissions. Further mentioning that soil cracking stimulated the greater emissions of N_2O whereas flooding irrigation regime had the least contribution (Huang *et al*., 2007).

Paddy soils are an important source of nitrous oxide emission, especially during frequent flooding and drying cycles. The N_2O flux from paddy soils is mainly driven by denitrifying microorganisms, but the response of denitrifying communities to flooding, drying cycles has been little studied (Liu *et al*., 2012). N_2O emissions were monitored under laboratory conditions in two paddy soils by Liu *et al*. (2012) and real-time qPCR and terminal restriction fragment length polymorphism (T-RFLP) were used to determine the abundance and community composition of narG and nosZ gene containing denitrifiers. N_2O emissions were significantly related to soil Eh than soil water content during drying process

indicating different responses among various denitrifiers in flooding and drying conditions. Copy number of narG increased with flooding and decreased with drying cycles. Liu *et al*. (2012) concluded that narG containing denitrifiers were much closely correlated with N_2O flux than nosZ containing communities in flooding drying conditions of soils used in their study.

The other laboratory study conducted by Liu *et al*. (2007) compared the fluxes of N_2, N_2O and CO_2 from no-till and conventional till soils under the same water, mineral N and temperature conditions. They showed that greater potential is for N_2O loss from non-till soils than conventional till soils and also from NH_4^+ type fertilizer caused gaseous loss only at 60% water filled pore spaces, indicating that with nitrification and subsequent denitrification under low moisture conditions. Avoiding wet soil conditions and applying NO_3^- form of fertilizer were suggested by Liu *et al*. (2007) to reduce the potential N_2O loss from arable soils.

The emission of N_2O from rice paddy soils is less than that from upland crop fields, which is probably due to complete nitrate-nitrite reduction to N_2, since rice paddy soil is known to have strong denitrifying activity. Under the flooded conditions in rice fields, hence the anaerobic conditions are being developed with submergence, the more denitrification reaction could be dominant. This would cause to produce N_2 emissions to be dominant over N_2O emissions with that of a complete denitrifying activity of microbial. According to (Pramanik *et al*., 2014), fluxes of N_2O emissions in the fallow season were influenced by N_2O emission potentials of soil aggregates and followed opposite trends as those observed during rice cultivation. Cover crop applications increased N contents in soil aggregates especially in smaller aggregates (< 250 μm), and that proportionately increased the N_2O emission potentials of these soil aggregates. Further, they concluded that the doses of cover crop applications for rice cultivation should not be optimized considering only CH_4, but also N_2O should be considered especially for fallow season to calculate total GWP. Mineralization of organic N increases NO_3^- - N concentration in soil, which are precursors for the formation of nitrous oxide (N_2O). However, Pramanik *et al*. (2014) mentions that N_2O is a minor greenhouse gas emitted from submerged rice fields and hence it is not often considered during calculation of total global warming potential (GWP) during rice cultivation.
On the contrary, nitrogen fertilizer rate is the best single predictor of N_2O emission from agricultural soils, which are responsible for about 50% of global anthropogenic flux, but it is a relatively imprecise

estimator according to Shcherbak *et al* (2014). However, accumulating evidence suggests that the emission response to increasing N input is exponential rather than linear, as assumed by Intergovernmental Panel on Climate Change methodologies. Their results suggests a general trend of exponentially increasing N_2O emission as N inputs are increased to exceed crop needs. N_2O response to N inputs grew significantly faster than linear for synthetic fertilizers and for most crop types (Shcherbak *et al*., 2014). Soil is the foremost source of N_2O emissions to the atmosphere, and approximately two- thirds of these emissions are generally attributed to microbiological processes including bacterial and fungal denitrification and nitrification processes, largely as a result of the application of nitrogenous fertilizers (Zhu *et al*., 2014).

The quality and management of winter crop residues can also affect soil N_2O emissions by modifying soil NH_4^+ and NO_3^- contents, which in turn are substrate for nitrification and denitrification, respectively (Khalil *et al*., 2004). These are the main processes related to the N_2O generation in soil (Khalil *et al.*, 2004). Legume species could exacerbate N_2O emissions from flooded rice production systems (Aulakh *et al*., 2001), but there is little evidence regarding the effect of different residue management systems on the emission of these greenhouse gases according to Shan *et al*. (2008). Crop residue management and the quality of cover crops on N_2O emissions in flooded rice systems have been studied by Tiago *et al*. (2011). They evaluated the influence of crop residue management and the type of cover crop residue on the CH_4 and N_2O emissions from a flooded Albaqualf soil cultivated with rice in a greenhouse. According to Tiago *et al*. (2011), crop residues in the soil surface lowered both emissions than that from soils where crop residues incorporated. The type of crop residue had no effect on CH_4 emissions, while the N_2O emission was observed higher with leguminous crop residue than from rye grass, but when only they were left on soil surface. The maintenance of cover crop residues on the soil surface mitigates nitrous oxide emissions as well as methane emissions from rice soils with flooded irrigation system. The incorporation of crop residues into the soil intensifies soil reduction at 20 cm depth anticipating the CH_4 emissions in comparison to the residues left on soil surface. For this reason, in addition to the supply of labile carbon, the subsurface soil layer is possibly the main source of CH_4, when crop residues are incorporated into the flooded rice soils. Tiago *et al*. (2011) further concluded that in flooded rice

systems, methane emissions are predominated over nitrous oxide emissions accounting for more than 90% of the partial global warming potential.

Production of both N_2O and CH_4 gases is a function of soil redox potential (Hou *et al*., 2000). The critical soil redox potential for N_2O and CH_4 production has been demonstrated in some laboratory studies shown to be below -150 mV for CH_4 and above +250 mV for N_2O (Wang *et al*., 1993). These critical soil redox potentials suggest that both these gaseous productions rarely occur at the same time. Accordingly, when soil redox potential is relatively high, soil nitrification takes place which results in N_2O production. N_2O is mostly produced through nitrate reduction when the soil become moderately reducing or when the nitrate diffuses into a zone that is less oxidized than the zone in which it is formed (Hou *et al*., 2000).

Soil redox potential becomes lower if there is sufficient soil organic carbon. This lower potential enables CH_4 formation to occur. Hou *et al*. (2000) suggests that if the redox potential of soil is kept above the level necessary to support CH_4 formation and kept below the moderately reducing condition where N_2O is formed, both gases could be minimized. This range was approximately between -100 mV and +200 mV which is too high to support CH_4 production, but low enough to favour N_2 production over N_2O production during the process of denitrification. Water management and organic carbon practices should be developed to maintain the redox potential at an intermediate range (around -100mV to +200mV), so as to minimize the production of these GHG emissions from flooded fields as suggested by researches (Hou *et al*., 2000).

However judging through previous researches, this range is proposed just to control N_2O which is a greenhouse gas, but it is not to control the complete denitrification process. This redox range favours the complete reduction of nitrate into nitrogen gas over nitrous oxide. However, it does not mean to be a solution for controlling the complete denitrification reaction in crop soils for improving soil nitrogen retention efficiency, especially in flooded rice soils. There is an emerging need to improve nitrogen fertilizer retention efficiencies in crop soils as well as with mitigating GHG emissions.

2.4 Controlling N losses in soil

2.4.1 Traditional solutions

The farming problems associated with excessively wet soils have led to placing pipes and ditches in many fields which reduces the potential for denitrification. Irrigation and farming practices for example, soil drainage and irrigation management, nitrogen fertilizer application rates and timings, selection of nitrogen fertilizer source, cover crops and residues (Kaewpradit *et al*., 2008), tillage and compaction practices are few traditional approaches associated with improving nitrogen retention efficiency in crop soils; as also discussed in the section 3.

2.4.2 Technical solutions

Slow releasing coated urea fertilizer, deep placement of fertilizer nitrogen, nitrification inhibitors, biological nitrogen fixation (BNF) attempts by using microbes are few examples of technical approaches associated with. The use of nitrification inhibitors has been consistently shown to lower denitrification and N_2O emissions. By slowing the conversion of ammonium to nitrate, there is less exposure of the N fertilizer to denitrification processes at any time. Microbial fuel cell applications are being currently investigated by our laboratory as an attempt to control denitrification in flooded rice soils though invoked redox changes in soil.

2.4.2.1 Paddy field microbial fuel cell

MFC applications possibly in cultivated land such as rice paddies is opposite to enhancing anaerobic digestion, but also finds in totally different settings, as also discussed by Arends and Verstraete, 2012. Accordingly, an example of a possible application is mitigation of the release of the greenhouse gas, methane. This greenhouse gas is produced under specific redox conditions. By adding electrodes (possibly inoculated with an active microbial community) the release of these greenhouse gases can possibly be prevented (Arends and Verstraete, 2012). However, since the production of methane and nitrous oxide gases from flooded paddy soils have trade off patterns (Hou *et al*., 2000), redox conditions in soils should also be controlled therefore to make a minimum of both gaseous productions accordingly.

Paddy field MFC is a sediment microbial fuel cell that fuelled by root exudates of paddy plants. An anode buried into the rhizosphere soil, connected to a cathode, lying in the overlaying water allows current production by its electrogenic micro-organisms. In a paddy field microbial fuel cell, electrogenically active bacteria (EAB) could involve in generating an electric current. Family *Geobacterracea* was abundant EAB in MFC of large currents and *Geobacter sulferreducens* were a well-characterized EAB with a high electricity generating capacity in the presence of acetate (Bond and Lovley, 2003). They were located on root surfaces and were more abundantly on granular graphite anodes. Anaerobic cellulytic bacteria of the families of *Clostridiacea* and *Ruminococcacea* were present in the areas where EAB were present, suggesting that current was generated via hydrolysis of carbohydrates into acetate by these families, and then to an oxidization of acetate carried out by *Geobacter sulferreducens*. Putative short chain fatty acid utilizing facultative denitrifying bacteria were abundantly detected in the low-current rhizosphere MFCs, and were speculated therefore as major competitors of EAB for electron donors. They were of *Rhodocyclacea* and *Commamonodacea* families. Acetate consuming methanogens were lower in high current MFCs, suggesting that EAB outcompete methanogens under conditions where anodes efficiently work as electron acceptors. These findings were also reviewed and explained by Watanabe and the group (2014), on recent advances and perspectives in rhizosphere microbial fuel cell. Arends *et al*., 2014 also studied greenhouse gas emissions from rice microcosms amended with a plant microbial fuel cell. Also, in accordance with Bond and Lovley (2003), when the current consuming bacteria outcompete the current producing bacteria closer to the anode, a less current output (voltage output) was probable with a paddy field-MFC.

Thus, our current studies are based on a hypothesis that electrogenically active bacteria could possibly outcompete the denitrifying bacteria in the rice soil systems. Especially, the electrogenically active bacteria could be rhizosphere-associated, due to the high availability of organic substances with its root exudates in the rhizosphere. Electrons released throughout the metabolic activities of these microbes, could yield a better MFC performance as well. Plant/microbe cooperation for electricity generation in a rice paddy field was studied by Kaku *et al*. (2008) while, MFC generating electricity from rhizo-deposits of rice plants was studied by Verstrate and the group (2008) also. Other than the purpose of harnessing electricity from MFC, we investigate on the applicability of paddy field MFC as a remedy

for controlling denitrification in flooded rice fields based on the soil redox changes that can be invoked by MFC.

Electrons are proposed to transfer via conductive nanowires (or structures called pili) in a long ranged electron transfer mechanism when biofilms are furthest from electrodes whereas the electrons transfer via membrane bound cytochromes when biofilms are closer to the electrodes as shown by Ashley *et al*. (2010). MFC engineering approaches of microbe - electrode conversion of organic matter to electricity and that of direct electron transfer from electrodes to microbes are also discussed by Lovley (2006), Lovley (2008), Lovley (2011). Also, Lovley and Nevin (2011) described new applications and concepts for microbe - electrode electron exchange to the Current Opinion in Biotechnology. However, beyond the main purpose of harnessing electricity, applications of MFC could be considered as a technical approach of controlling denitrification in rice soils, yet the theory warrants future research insight for a field scale development.

CHAPTER 3

STUDY OF NITROGEN BEHAVIOR IN MICROBIAL FUEL CELL APPLIED RICE SOILS

3.1 Introduction

Denitrification, which converts soil nitrogen to nitrogen gas, involves a great loss of nitrogen fertilizer. Controlling the denitrification rate could help reduce the losses of applied nitrogen fertilizer in fields. The applicability of microbial fuel cells for controlling denitrification in flooded rice soils was investigated based on MFC theory coupled with redox changes. Because the soil about 10–20 cm beneath water and the soil near the water surface are anaerobic and aerobic, respectively, potential gradients could be generated between them, upon connecting them through insulated wires. Electrons released through the oxidation of organic matter during microbial metabolism can be utilized by this set-up, generating electricity. This can reduce the availability of electrons for reductive half-reactions of nitrate, to suppress denitrification. We studied the nitrogen behavior in soil using a planting pot with gas chamber experiment under three conditions: MFC systems, MFC systems with an externally applied voltage, and non-MFC systems as a control. The MFC systems with an externally applied voltage were aimed at increasing the efficiency of MFCs because it was expected that the rapid movement of electrons towards the anodic area would be enhanced by the externally applied voltage. In the rest of this article, we refer to the MFC systems as MFCs, MFC systems with an externally applied voltage as MFC-extVs, and non-MFC systems as non-MFCs.

3.2 Materials and methods

3.2.1 Design of planting pots and gas chamber experiments

An external voltage maintained at 25–50 mV was applied by a voltage stabilizer (AD-8735A; AND Company Ltd.) throughout rice growth. The experimental period included the entire period of rice growth after transplantation. Soil redox potential, N_2O gas flux, C/N ratio of soil, and NH_4^+, NO_2^-, and NO_3^- concentrations in soil pore water were periodically measured. Based on these measurements, the nitrogen retention efficiencies and denitrification loss rates were calculated and compared among these systems.

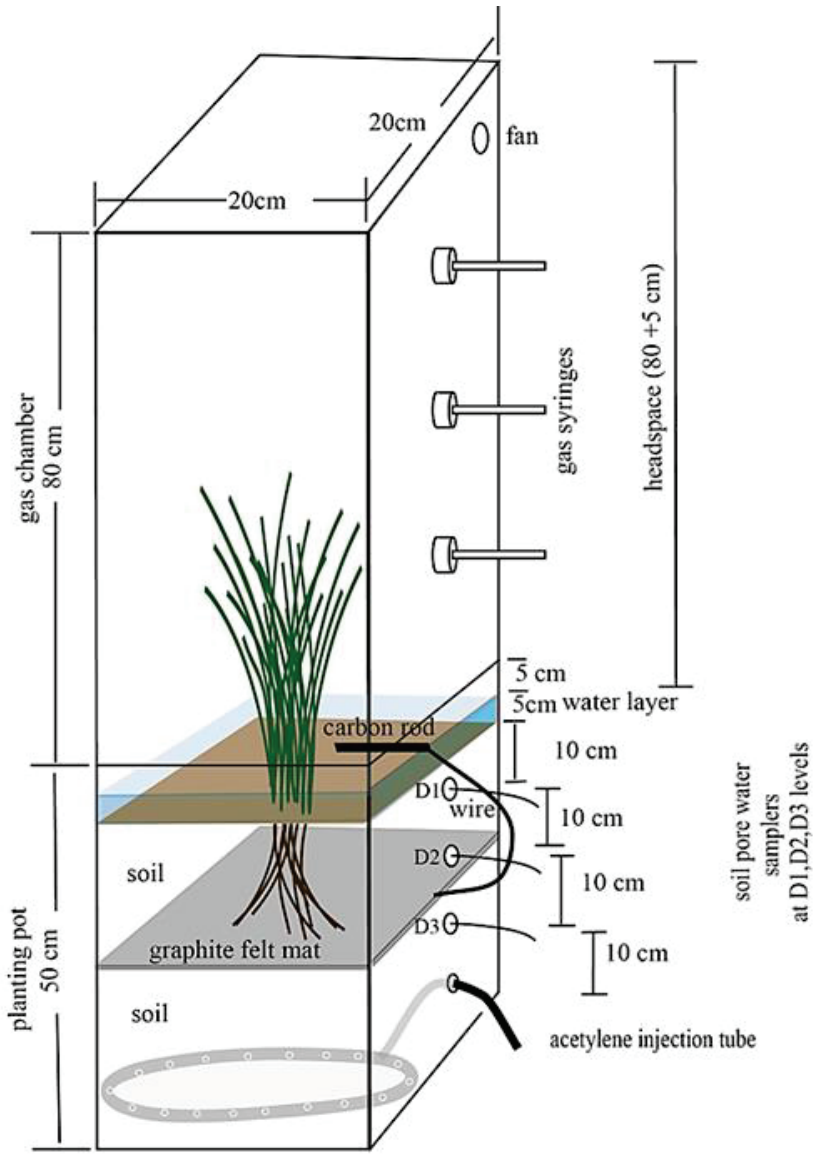


Figure 3.1 Design of gas chamber and planting pot. Headspace is defined as the height of the chamber plus the distance between flooded water surface and the top of the pot.

The designs of the planting pot and the gas chamber are shown in **Figure 3.1**. For soil particle and pore water samplings, each planting pot had three holes (diameter of 2 cm) closed by rubber stoppers. In addition, each planting pot had a hole at the bottom, to inject acetylene gas in order to inhibit the reaction of nitrous oxide reductase enzymes, which further converts N_2O to N_2. A circular tube, which had holes in it at equal distances of 2.5 cm from each other, was fit snugly into the bottom hole and then inserted into soil. Because this tube was connected to a three-way stopcock, gas could be distributed at

equal pressure in each pot. Gas flow pressure at the outflow valve was adjusted to 1.2 MPa. Each gas chamber also had three holes (diameter of 2 cm) closed by a butyl rubber septum for gas sampling.

Table 3.1: Basic soil physical properties

Bulk density (g/cm^3)	Dry density (g/cm^3)	Soil porosity (%)	Soil water content (%)
1.04	0.82	60.7	27

The soil for the experiment was collected from the experimental paddy fields of the Field Science Center of Gifu University. Some of the soil was used for determination of its physical properties (**table 3.1**), while the rest was filled into pots. Except for in the non-MFCs, for an anode, a carbon graphite-felt mat (S-221; Osaka Gas Chemicals Co., Ltd.) of 20 × 20 × 0.5 cm was introduced 10 cm below the soil surface. As a cathode, a graphite rod (C-072591; Nilaco Corporation) with a length of 10 cm and diameter of 0.5 cm was kept floating on the flooded water in contact with air. The anode and cathode were linked through insulated wires externally connected with a resister of 330 Ω.

As a basal application, ammonium-based chemical fertilizer $(NH_4)_2SO_4$ was initially applied at a rate of 30 kg Nha^{-1}. One day after the application of fertilizer, two rice seedlings (aged 3 weeks) were transplanted into each pot on June 15th, 2016. Then, soil was kept flooded at a depth of approximately 5 cm by an automatic water supply system. Rice growth can be divided into three stages: vegetative, reproductive, and maturation. June 24 was considered as the start of the vegetative stage with the tillering of rice plants. Panicle forming of rice plants first visibly started on July 16, which was considered as the start of the reproductive stage. Heading time started on August 16, which was considered as the start of the maturation stage.

3.2.2 Gas sampling and measurements

Applying a closed chamber method, gas was sampled biweekly and N_2O concentration was measured by gas chromatography (GC-2014 equipped with an ECD detector; Shimadzu Co., Ltd.). Using an injecting syringe in each sampling hole of the chamber, gas was sampled at 11 a.m., 1 p.m., and 3 p.m. at each gas sampling event. The reason why N_2O concentration was measured is that the measurement

of N_2 emissions is impossible due to the high atmospheric background level of 78% (Groffman and Robertson, 2015). To quantify the rates of denitrification by microbial processes accurately, reduction of N_2O gas into N_2 should be prevented. Thus, the acetylene inhibition method was applied (Iida et al., 2007). Forty minutes before the start of gas sampling, acetylene gas was injected for 1 min, to achieve a state of equilibrium between the soil and the chamber headspace. Iida et al. (2007) proposed that the amount of acetylene in the headspace needs to be higher than 0.1% to ensure that microbial enzymes related to N_2O reduction to N_2 are inhibited. According to this standard, the acetylene concentration in the headspace was checked by tube-type gas detectors (GV100S; GASTEC Co., Ltd.) before starting the sampling and at the last sampling. Before each gas sampling event, a small fan fixed on the top corner of the chamber was operated for 30 s to ensure the homogeneity of the air inside the chamber. The conditions of gas chromatography were as follows: oven temperature: 60°C, detector temperature: 340℃, and carrier gas: argon + methane (5%). The unit of concentration was converted from volumetric concentration to mass concentration using the ideal gas law, considering headspace temperature and assuming gas pressure of 101,325 Nm^{-2}. Then, flux was calculated using the following equation.

$$F = \frac{\Delta m}{A \cdot \Delta t} = \frac{V}{A} \cdot \frac{\Delta m}{V \cdot \Delta t} = h \cdot \frac{\Delta m / V}{\Delta t} \quad (3)$$

Here, F is N_2O flux ($\mu g\ m^{-2}\ min^{-1}$), Δt: measured time (min), Δm: mass change of the N_2O gas during Δt, A: surface area of the chamber (m^2), V: volume of headspace (m^3), and h: effective height of headspace (m). Here, the headspace is defined as the height of the chamber plus the distance between the flooded water surface and the top of the pot **(Figure 3.1).**

3.2.3 Redox potential and chemical measurement

Redox potential was measured by Eh meters (PRN-41; Fujiwara Factory Co., Ltd.). These meters consist of counter electrodes made of Pt coated by glass tubes, and reference electrodes made of AgCl with saturated KCl internal solution. While reference electrodes were put near the soil surface, counter electrodes were installed at a depth of 20 cm, just near the anode, and continuously measured Eh at 1-

h intervals. The voltage difference generated by the MFCs and the soil temperature (at 10 cm below the soil surface) and the temperature inside the chamber were recorded on a data logger (CR-1000; Campbell Scientific Inc.) at 10-min intervals.

Soil pore water was sampled on June 24 (the start of the vegetative stage), July 22 (the start of the reproductive stage), and August 9 (the start of the maturation stage), using pore water samplers (RHIZON MOM 10 cm; Rhizosphere research products, www.rhizosphere.com). Sampling depths were 10, 20, and 30 cm from the soil surface. Upon filtering through membrane filter units of 0.45-μm pore size (03CP045AN; ADVANTEC) and then through membrane filters of 0.45-μm pore size (A045A047A; ADVANTEC), NH_4^+, NO_2^-, and NO_3^- concentrations of filtrates were identified by ion chromatography (ICA-2000; TOA-DKK Co., Ltd.). The eluents of ion chromatography were 1.8 mM Na_2CO_3 and 1.7 mM $NaCO_3$ for anions and 6 mM methanesulfonic acid for cations.

Total carbon and total nitrogen contents in soil particles were measured by a CHNO elemental analyzer (JM10 micro-corder; J-SCIENCE LAB Co., Ltd.). Soil sampling was carried out at the same time as gas sampling and pore water sampling, so that the soil environment would not be repeatedly disturbed. Sampled soil was air-dried for 48 h and sieved by a 2-mm sieve.

3.3 Data analysis

3.3.1 Data treatment and statistical analysis

After initiating the experiment, MFC output was not stabilized for some period. Thus, the data obtained after June 23 were utilized for analysis. In addition, the redox potential showed periodical drops due to acetylene injection during the gas sampling events. Thus, redox potential data during the gas measuring events were excluded from the analysis. Using daily average redox potential and N_2O flux, we compared the difference between the treatments. In addition, by conducting statistical analysis such as one-way ANOVA and multiple comparison, differences between treatments were statistically examined. All statistical analyses described in this article were performed using IBM SPSS Statistics version 24.

3.3.2 C/N ratio

The examination of redox potential and N_2O flux might provide useful information on the effects of MFCs and MFC-extV. In addition, as for supportive data, we also examined the C/N ratio of soil particles. Because the availability of electron donors plays an important role in denitrification, the C/N ratio can be used as a kind of index of denitrification activity. This means that a low C/N ratio limits the electron supply for reductive half-reactions, thereby leading to the accumulation of denitrification intermediates, NO_2^-, NO, and N_2O (Krishna Mohan et al., 2016).

3.3.3 Nitrogen retention and denitrification

Based on the observations of low NH_4^+ - nitrogen ions in soil particles in random samples (of soil solutions with a 1:2 ratio), NH_4^+ - nitrogen retention in soil particles was considered negligible. In addition, we assume that N mineralization and immobilization are slower in flooded conditions due to the low oxygen levels, compared with those in upland soils (Ishii et al., 2011). Therefore, the contributions of mineralization and immobilization to the N budget are considered negligible. By calculating the mass change of inorganic nitrogen in the soil pore water, the net change of inorganic nitrogen can be estimated. Because soil sampling depths are 10, 20, and 30 cm, we divided the pot into three layers with heights of 15, 10, and 15 cm, respectively, as shown in **Figure 3.2**. It could be considered that the whole soil pore spaces were fully saturated over the period, since the soils were continuously flooded. Thus, we can use soil porosity to calculate the volumetric water content of each layer. Then, if the amounts of inorganic nitrogen in the forms of NH_4^+, NO_2^-, and NO_3^- of each layer are summed up, we can obtain the total inorganic nitrogen amount of soil pore water by using the following equation (4).

$$TN = \sum_{i=1}^{3} C_{N,i} \cdot V_i \cdot \theta_i \quad (i=1, 2, 3) \tag{4}$$

Here, TN is total inorganic nitrogen amount (mg), i: index representing the layer number, $C_{N,i}$: total inorganic nitrogen concentration at the ith layer (mg l^{-1}), V_i: soil volume of the ith layer, and Θ_i: soil porosity of the ith layer.

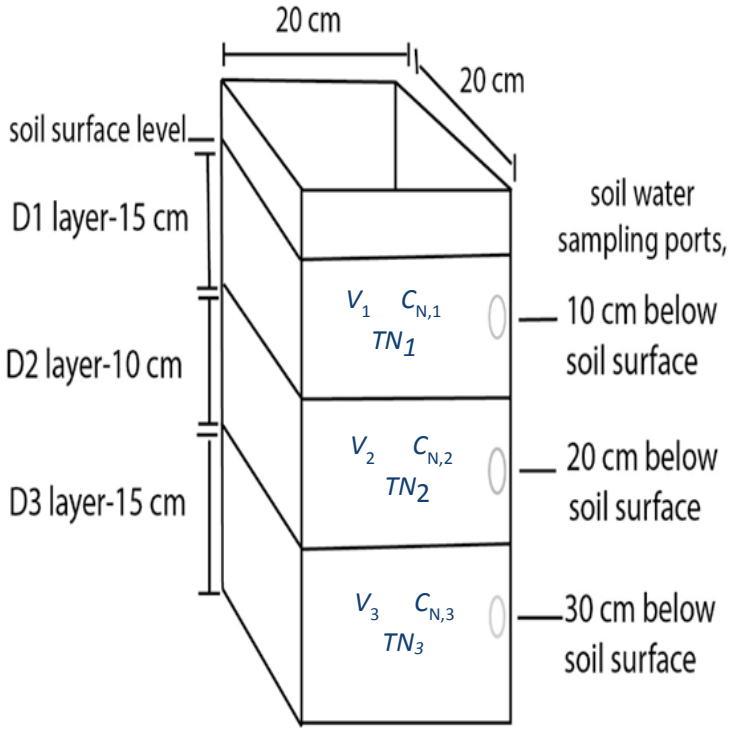


Figure 3.2 Sketch of soil depth profiles used for inorganic N calculations

Then, inorganic nitrogen retention efficiencies defined as the ratio of *TN* of each growth stage to the initially applied amount of nitrogen fertilizer were calculated. At the same time, measured N_2O flux can be used to estimate the denitrification rate of each system. The average rate of nitrogen flux of each rice growth stage was used to estimate the total denitrified nitrogen loss in relation to the specific growth stage using the following equation.

$$DN_{(growthstage)} = 1440 \cdot N_{flux(growthstage)} \cdot A \cdot d_{(growthstage)} \quad (5)$$

Here, $DN_{(\text{growth stage})}$ is denitrified nitrogen amount in each growth stage (mg), $N_{flux\ (\text{growth stage})}$: average nitrogen flux in each growth stage (μg m^{-2} min^{-1}), A: surface area (m^2), and $d_{(\text{growth stage})}$: number of days of each growth stage. By summing up the losses of each growth stage, total denitrified nitrogen as a percentage of initially applied nitrogen amount was estimated for each system.

3.4 Results and discussion

3.4.1 Redox potential and N_2O flux

Time series of the daily average redox potential of each treatment are shown in **Figure 3.3**. Both MFC and MFC-extV treatments showed significantly higher redox potentials than that of non-MFCs according to the multiple comparison of Turkey HSD tests s after one-way ANOVA (**Figure 3.4**). During the reproductive stage, there were distinct differences between MFCs and non-MFCs. However, during the maturation stage, redox potential reached between −180 and −200 mV in each system, and showed similar values irrespective of the MFC effects. Electrode malfunction might be one reason for this. Another possible explanation is the maturation of rice plants, at which stage the exudation of organic acids from the rhizosphere might be limited. Quantitative and qualitative differences in the exudation of organic substrates from rice roots would have affected the MFC performance, which was also discussed by Kaku et al. (2008). In fact, according to Aulakh et al. (2001), root exudation rates generally increase with seedling to panicle initiation or flowering, but decrease at maturity. We thus speculated that the reproductive stage of rice could facilitate root exudation. Therefore, with the high availability of electrons provided by root exudates (i.e., organic acids), the performance of MFC could be higher, with the circulation of more electrons released with the consumption of organic exudates due to microbial metabolism. This in turn could have a greater effect on controlling the soil redox status during the reproductive stage of rice.

N_2O flux over the experimental period with each treatment is shown in **Figure 3.5**. Again, one-way ANOVA on N_2O flux data showed a significant difference between each treatment at the 5% significance level. A Tukey HSD multiple comparison test was applied to compare treatments, the results of which are shown in **Figure 3.6**. While N_2O flux showed a significant difference between MFCs and non-MFCs, there was no statistically significant difference between MFCs and MFC-extV.

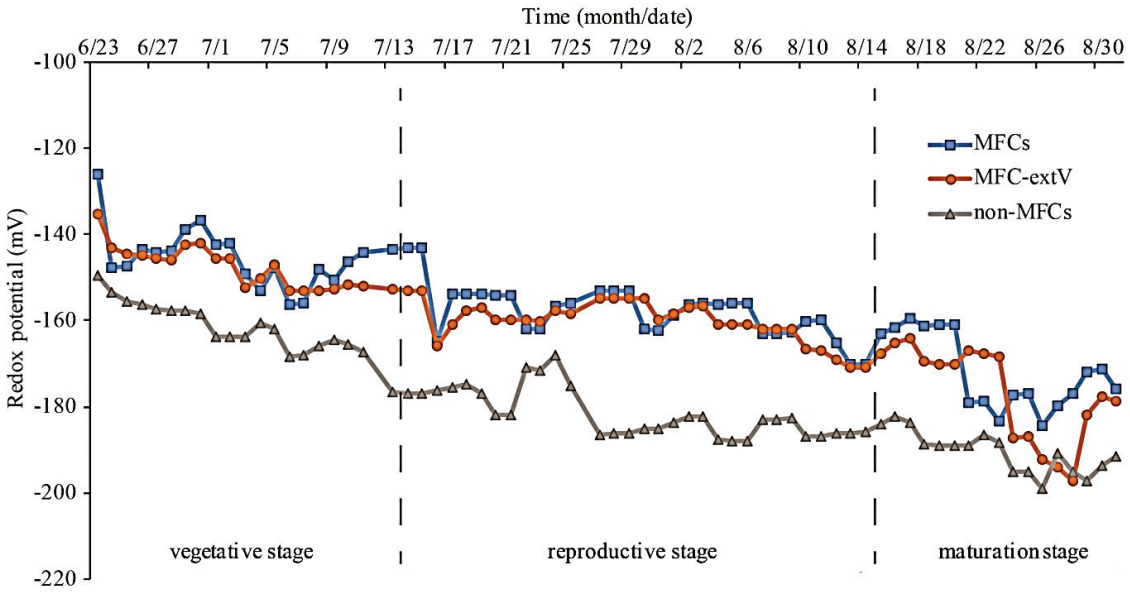


Figure 3.3 Soil redox pattern over the experimental period

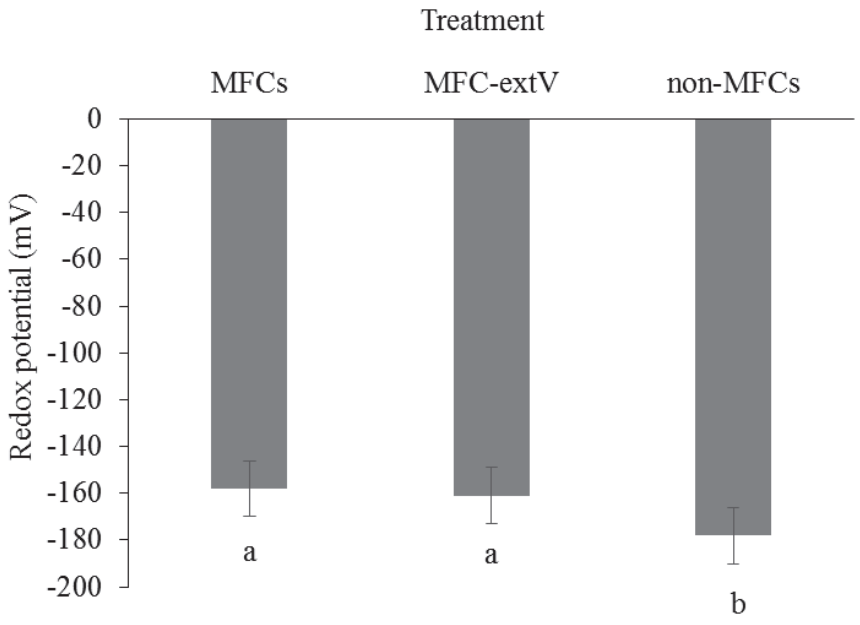


Figure 3.4 Results of multiple comparison of redox potential. Mean ± S.D. redox potential of soils in three different treatments are shown. Results of Tukey test are shown as letters below the columns, in that there are significant differences in the means of columns not sharing the same letter.

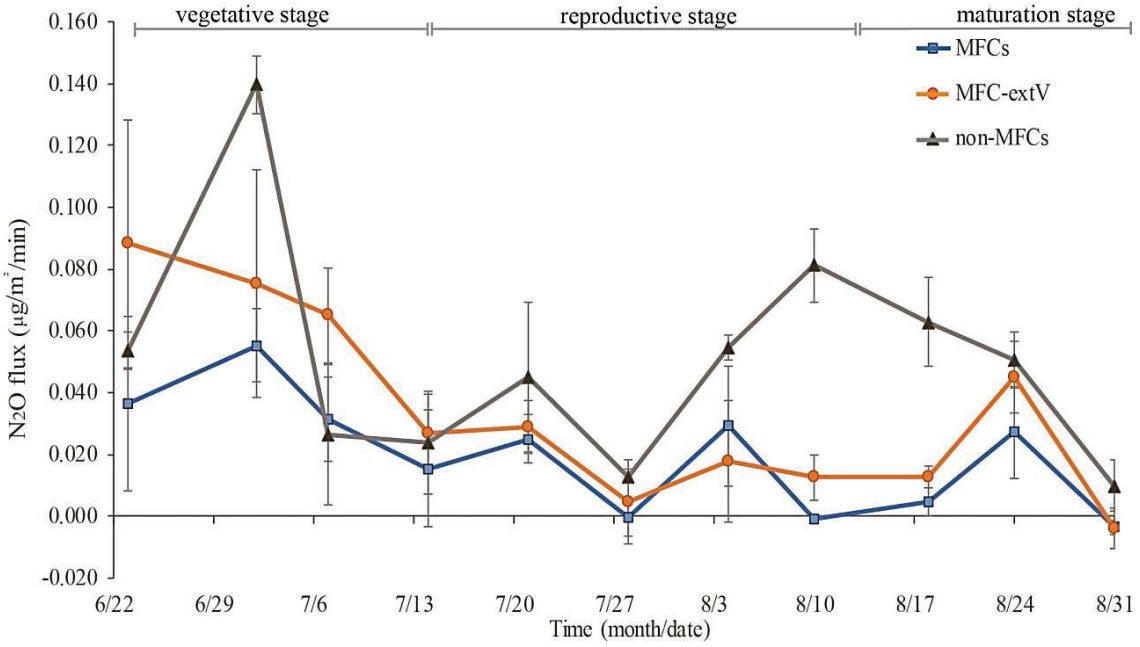


Figure 3.5 N_2O flux over the experimental period. Mean flux for each treatment measured over the whole period is shown. Bars represent standard deviation of the mean.

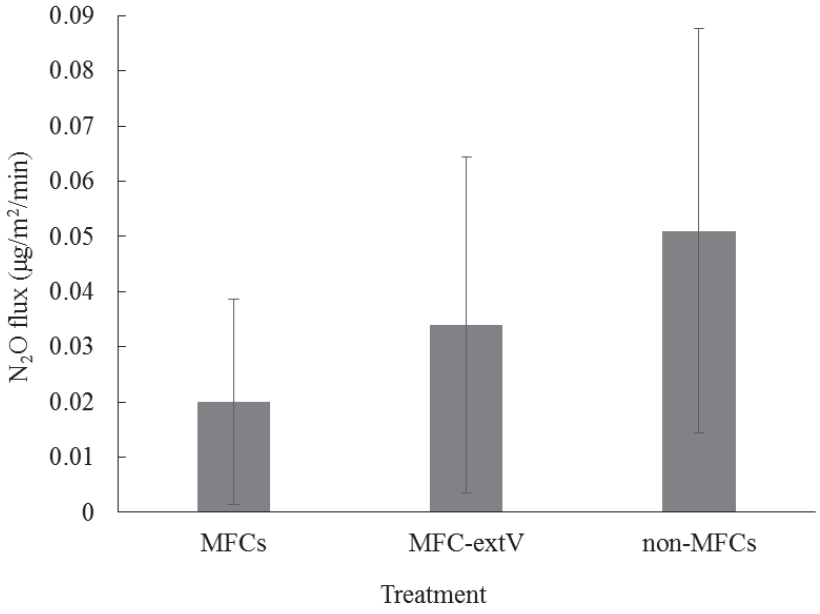


Figure 3.6 Results of multiple comparison of N_2O flux. Mean ± S.D. N_2O flux of soils in three different treatments are shown. Results of Tukey test are shown as letters below the columns, in that there are significant differences in the means of columns not sharing the same letter.

Both mean N_2O flux and average redox potential with respect to each growth stage are shown in **Figure 3.7**. There was a common trend of N_2O flux in all treatments, namely, a decrease from the start to the end of rice growth. However, N_2O flux of non-MFCs was significantly higher than those of MFCs and MFC-extV throughout all stages. While N_2O flux rates of non-MFCs were 0.078, 0.044, and 0.025 $\mu g/m^2/min$ in the vegetative stage, reproductive stage, and maturation stage, respectively, those for MFCs and MFC-extV were as follows: for MFCs, 0.045, 0.017, and 0.002 $\mu g/m^2/min$, and for MFC-extV, 0.052, 0.029, and 0.014 $\mu g/m^2/min$.

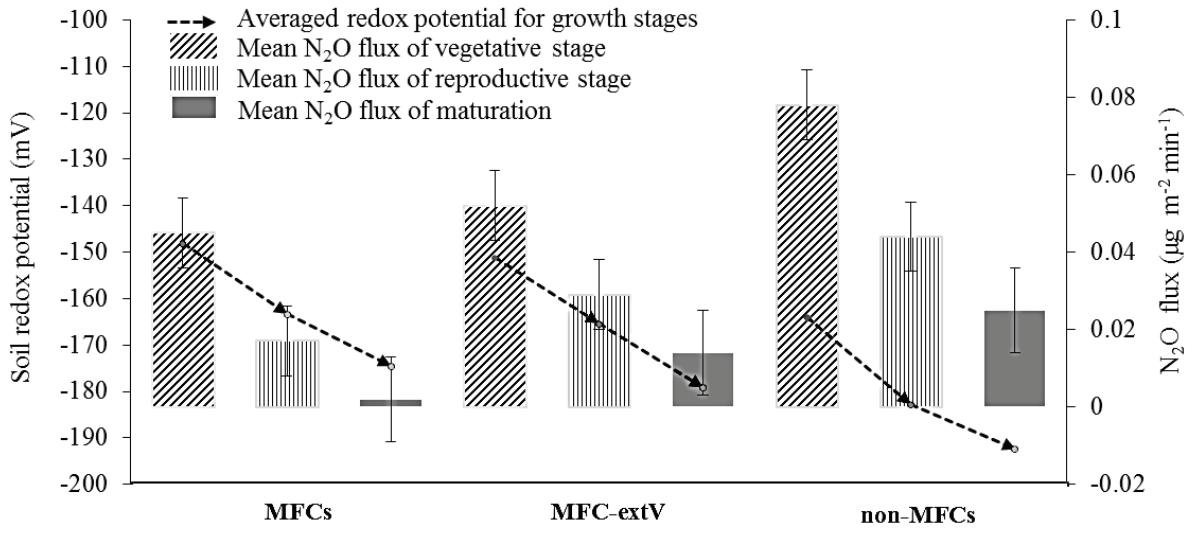


Figure 3.7 Overlay of average N_2O flux rates and soil redox potentials with treatments.

If we consider the redox potentials, in all treatments, they also showed a decreasing trend from the start to the end of rice growth. However, this decreasing trend was much more pronounced in non-MFCs. In addition, soil redox status in non-MFCs was far more reducing throughout all growth stages, namely, −164 mV, −183 mV, and −192 mV in the vegetative stage, reproductive stage, and maturation stage, respectively. By contrast, the redox conditions of MFCs and MFC-extV were as follows: for MFCs, −151 mV, −165 mV, and −179 mV, and for MFC-extV, −148 mV, −163 mV, and −175 mV, respectively.

Overall, these findings can be interpreted as follows. As a consequence of relatively high redox potential in MFCs and MFC-extV, denitrification processes are suppressed, and as a result N_2O flux is

also suppressed. This indicates that MFC systems have the potential to control the denitrification process.

3.4.2 C/N ratios in soil

The C/N ratios are shown in **Figure 3.8.** These ratios showed a tendency to increase over the growth period, irrespective of the treatment applied. Specifically, the MFCs were associated with lower C/N ratios than the non-MFCs during the reproductive stage and maturation stage. This implies that the availability of electrons for denitrifying half-reactions in the MFCs might have been limited, whereas the availability of electrons for denitrifying redox reactions in the non-MFCs was much higher. With regard to non-MFCs, while the C/N ratio in MFC-extV had an increasing trend over time, it was slightly higher than that of non-MFCs on August 9th.

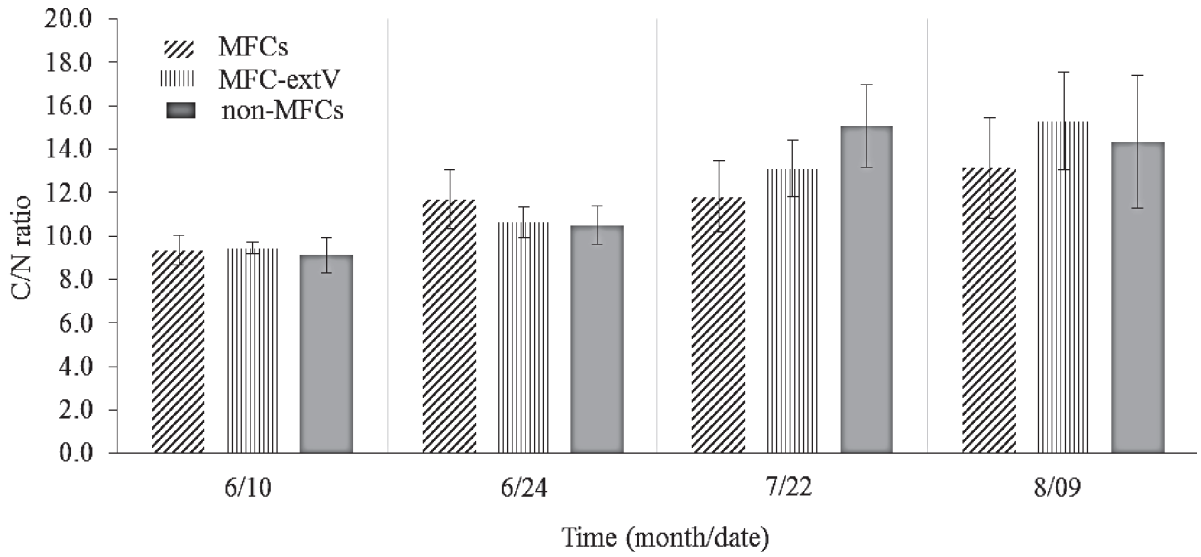


Figure 3.8 Behavior of total C/N ratio in soil. Mean C/N ratios for each treatment over the start of the rice growth stages are shown. Bars represent standard dev. of the mean.

3.4.3 Inorganic nitrogen profile

Inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-) profiles for each treatment at the three different times are shown in **Figure 3.9**.

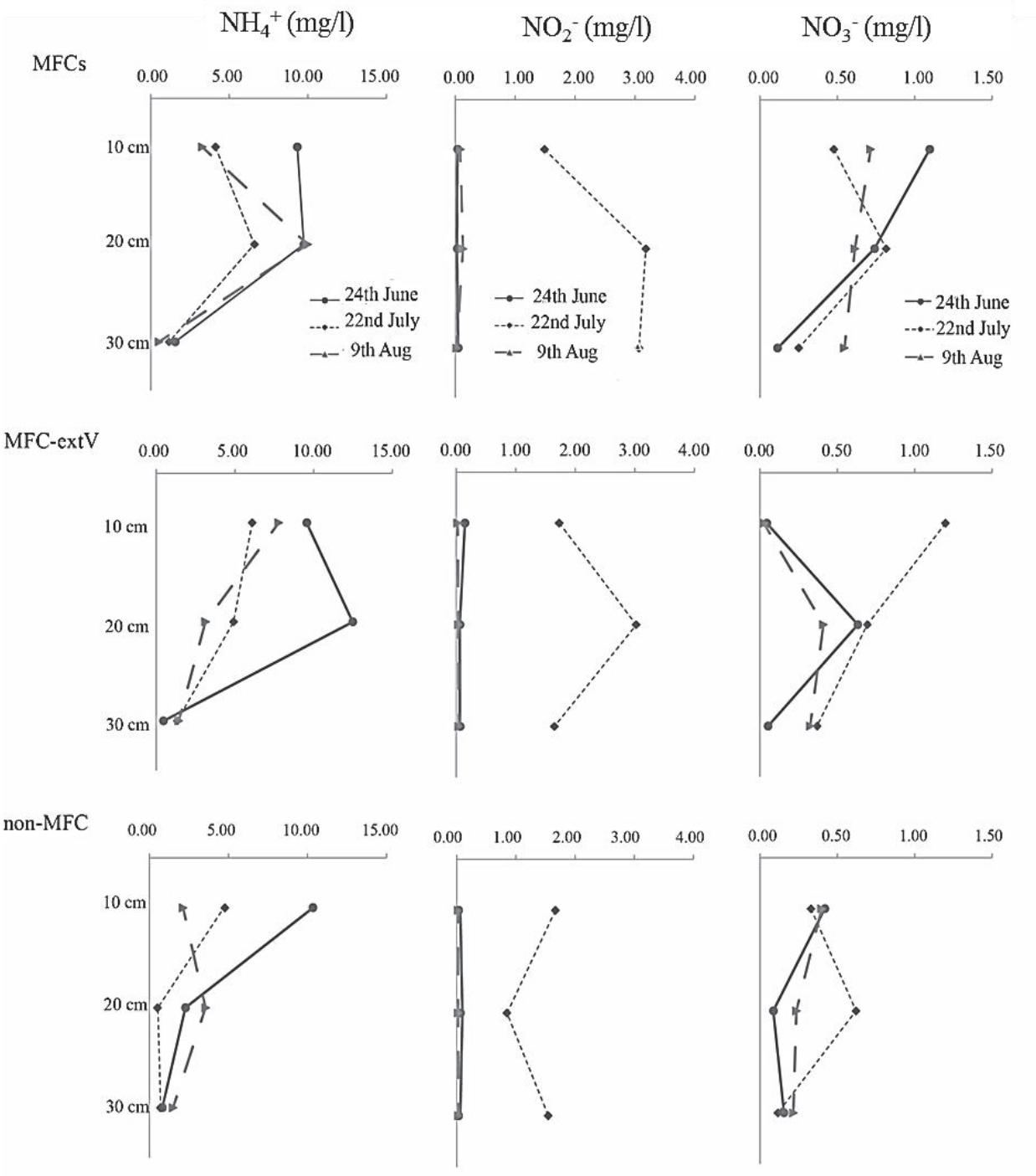


Figure 3.9 NO_2^-, NO_3^-, and NH_4^+ ion concentrations in soil pore water sampled at three different soil depths

Most of the inorganic nitrogen was localized in the upper soils at depths of 10 cm and 20 cm. NH_4^+ concentration at a depth of 10 cm was the highest at the start of the vegetative stage (June 24) in all treatments with the addition of fertilizer, but the size of the decrease was much larger in non-MFCs at the start of the maturation stage (Aug 9). While NH_4^+ concentration at a depth of 30 cm showed no significant difference among the treatments, especially the NH_4^+ concentration of MFCs at a depth of 20 cm was clearly higher throughout the growth stages.

In all three systems, relatively high NO_2^- concentrations appeared for a short time during the start of the reproductive stage. In general, for all three systems, this might have been attributable to the nitrification reactions by nitrifiers under aerobic conditions provided with fertilizer with high levels of nitrogen at the beginning of this experiment. This is referred to as a "nitrifier-denitrification" reaction because it is a nitrification pathway involving the oxidation of NH_3 to NO_2^-, followed by the reduction of NO_2^- to N_2O and N_2 (Wrage et al., 2001).

Accordingly, subsequent reductions can be expected due to the formation of these NO_2^- ions first and then their conversion into NO_3^- ions. However, it is expected that reductions are controlled in MFC systems. According to **Figure 3.9**, NO_2^- and NO_3^- ions were rarely present in non-MFCs during the start of the reproductive stage, suggesting that more denitrification reactions were likely with non-MFCs. Specifically, during the start of the reproductive stage, in both MFCs and MFC-extV, NO_2^- concentrations were higher than those of non-MFCs. This implies that denitrifying conditions had not occurred much in both MFC systems at this stage. However, towards the maturation stage, NO_3^- concentrations were lower in non-MFCs than in MFCs, implying that denitrification reactions are more likely to occur in non-MFCs than in MFCs. At the same time, these results are compatible with the N_2O flux changes in MFCs and non-MFCs, especially with the clear difference between them through the maturation stage **(Figure 3.5).**

3.4.4 Nitrogen retention and denitrification

The total amount of inorganic nitrogen in all soil pore water at the start of each growth stage is shown in **Figure 3.10.** During each stage, total inorganic nitrogen in MFCs was higher than that in non-MFCs. Interestingly, the availability of inorganic nitrogen was specifically higher in MFCs and MFC-extV during the start of the reproductive stage than at other stages.

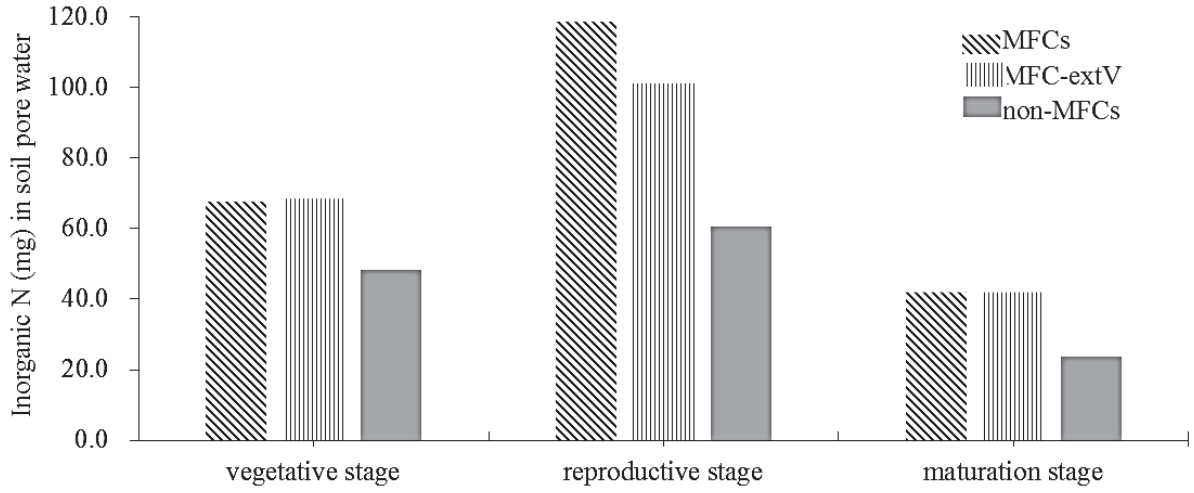


Figure 3.10 Total inorganic N variation in soil pore water

According to equation (4), the nitrogen retention efficiencies for MFCs, MFC-extV, and non-MFCs relative to the start of the reproductive stage were 98.8%, 84.2%, and 50.5%, respectively. By the start of the maturation stage, nitrogen retention efficiencies were 34.9%, 34.9%, and 19.9% with each system, respectively. This could be explained by the fact that the retention efficiencies decrease during the period of growth, with a diminishing source of nitrogen, and also with nitrogen utilization by plants. Accordingly, both MFC conditions showed relatively high efficiencies, especially at the reproductive stage of rice growth, as shown in **Table 3.2**.

Total denitrified N losses as calculated by equation (5) are also shown in **Table 3.2**. Total denitrified N levels by the end of the maturation stage of rice estimated for the systems of MFCs, MFC-extV, and non-MFCs were 2.8%, 4.0%, and 6.1%, respectively. Accordingly, both MFCs and MFC-extV showed lower denitrification N losses than non-MFCs.

Table 3.2 Inorganic nitrogen retention efficiencies and denitrification rates

	Rice growth stage	TN retention efficiency (%)	N_2O flux ($\mu g/m^2/min$)	Denitrified N (%)
MFCs	vegetative	56.3	0.045	2.8
	reproductive	98.8	0.017	
	maturation	34.9	0.002	
MFC-extV	vegetative	56.9	0.052	4
	reproductive	84.2	0.029	
	maturation	34.9	0.014	
non-MFCs	vegetative	40.2	0.078	6.1
	reproductive	50.5	0.044	
	maturation	19.9	0.025	

3.4.5 Reasons for lower denitrification rates

In other studies (Groffman et al., 1995; Pathak et al., 2007), the denitrification rate from paddy soils was estimated to be in the range of 10%–50%. Thus, the results of our study show a comparatively low rate, although there was a significant difference in denitrification rate between MFCs and non-MFCs. Two possible reasons for this are as follows.

While our desired inhibition by the acetylene inhibition method involves the suppression of nitrous oxide reductases that further reduce N_2O into N_2 gas, acetylene gas also inhibits the production of NO_3^- via nitrification (Mosier, 1980; Groffman et al., 1995). This is undesirable for a denitrification study with NH_4^+ fertilizer because NH_4^+ ions could not be denitrified unless nitrification occurs first. Therefore, there is a possibility that a significant proportion of NH_4^+ ions were not converted to nitrate, which led to underestimation of the overall denitrification rate.

Another possible reason is that the amount of N_2O gas emitted into the air from flooded rice soils could decrease due to the amount of it dissolved in the flooded water layer. As we sometimes observed negative N_2O flux, as shown in **Figure 3.5**, this could be another reason for the lower denitrification rates.

3.5 Conclusion

We attempted to confirm the applicability of MFC theory for suppressing denitrification in soils. From the nitrogen behavior studies in soils of planting pot with chamber experiments, we obtained the result that N_2O flux levels of MFCs and MFC-extV were significantly lower than that of non-MFCs. Both redox potential trends and the C/N ratio in MFCs and MFC-extV were also consistent with this result. More specifically, the results showed that the effect of MFC could be better utilized in the reproductive stage. This period is the most critical stage throughout rice growth because better availability of nitrogen is required during this period. However, whether the external application of voltage has distinct effects on redox potential and N_2O flux rate was not clarified. This might be attributable to the design of the anode. Therefore, additional consideration of anode design should be performed to cover the effect of external voltage through the soil.

During the initial stage of this experiment, the effect of MFC was not clear. This may have been attributable to the overlap of several mineral nitrogen transformations at this stage, including the autotrophic nitrification process, irrespective of MFC application. NH_4^+ - nitrogen, the form of nitrogen usually applied as fertilizer, is first converted to NO_2^- and then to NO_3^- under aerobic conditions. However, these nitrification reactions might be partially blocked by frequent acetylene injections, which corresponded to the underestimation of denitrification rates in all systems. Nonetheless, total inorganic nitrogen retention efficiencies in soil pore water suggested that best retention occurred in the reproductive stage of rice growth with MFC systems. The total denitrified nitrogen percentage by the end of the maturation stage suggested that the denitrification reaction was controlled in the MFC systems. In conclusion, we confirmed the applicability of MFC to control soil redox potential and thereby suppress denitrification, namely, nitrogen losses, in flooded rice fields. This approach could also lower greenhouse gas (N_2O) emissions. Further studies are required to assess denitrifying conditions by monitoring the activity of denitrifying microbes and their population shifts associated with MFC treatments.

CHAPTER 4

SOIL REDOX DISTRIBUTION PATTERN IN RICE SOIL PROFILES WITH MICROBIAL FUEL CELL PLUS EXTERNAL VOLTAGE APPLIED

4.1 Introduction

External voltage was applied in the aim of increasing microbial fuel cell efficiencies, for the better control of soil redox potential. An externally applied voltage, ranged at 25-50 mV, was aimed at conveying more electrons towards the anodic area for the efficiency of MFC. Therefore, the denitrification rates were expected to be lower, compared to that of MFCs. However, the redox controlling potentials and denitrification controlling potentials of MFC-extV systems were lower than that of microbial fuel cell systems. Therefore, in this study, soil redox distributions at three depth profiles over the period were compared in both systems and controls. It was hypothesized that the redox distribution patterns with external voltage applications were not effected through whole soils, but were effected locally at the points of MFC anodes where they were connected through external voltage. In this study, we investigate the effect of external voltage applied to MFCs on redox distributions at different soil depth profiles.

4.2 Materials and Methods

4.2.1 Design of the experiment

For studying the Eh - depth profiles of soils, pot experiment was conducted under the three conditions: MFC systems, MFC systems with an externally applied voltage, and non-MFC systems as a control. All the soil systems monitored were not plant-assisted in this experiment. The design of the soil Eh-depth profile study with potted soil is shown in **figure 4.1**. Redox potential was measured by Redox (Eh) meters (PRN-41; Fujiwara Factory Co., Ltd.). Eh meters consist of counter electrodes made of Pt coated by glass tubes, and reference electrodes made of AgCl with saturated KCl internal solution. While reference electrodes were put near the soil surface, counter electrodes were installed at the center, and at both sides 5 cm away from the center at each soil depth levels of 10 cm, 20 cm and 30 cm from the surface soil. Eh was continuously measured at 2 days' intervals. The voltage difference generated by the MFCs and the soil temperature (at 10 cm below the soil surface) were recorded on a data logger (CR-1000; Campbell Scientific Inc.) at 10-min intervals.

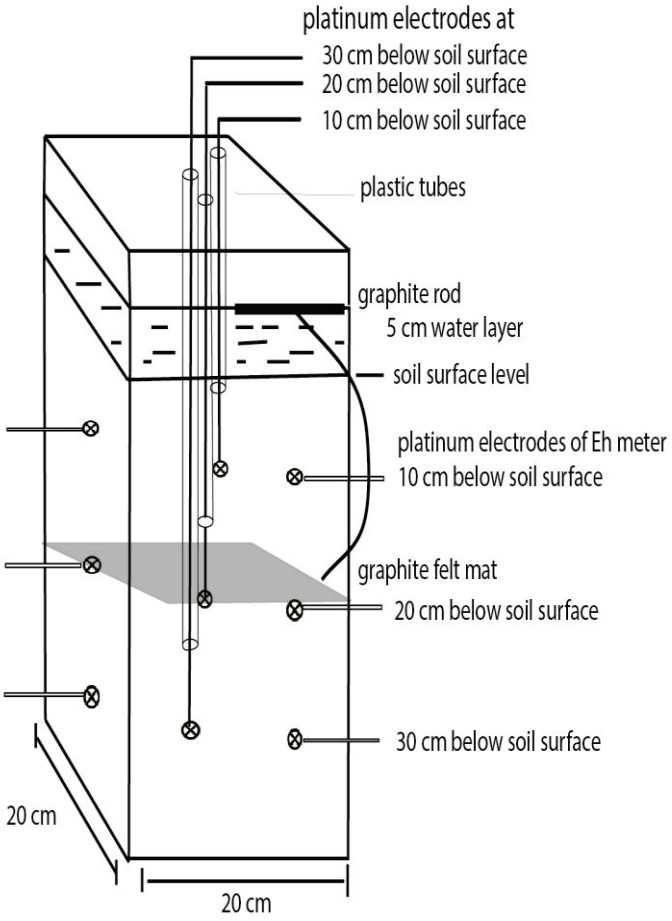


Figure 4.1: Design of the soil Eh-depth profile study with MFC treatments and controls

The soil for the experiment was collected from the experimental paddy fields of the Field Science Center of Gifu University and was filled into pots. Except for in the non-MFCs, for an anode, a carbon graphite-felt mat (S-221; Osaka Gas Chemicals Co., Ltd.) of 20 × 20 × 0.5 cm was introduced 20 cm below the soil surface. As a cathode, a graphite rod (C-072591; Nilaco Corporation) with a length of 10 cm and diameter of 0.5 cm was kept floating on the flooded water in contact with air. The anode and cathode were linked through insulated wires externally connected with a resister of 330 Ω. After the MFC configurations, soil was kept flooded at a depth of approximately 5 cm by an automatic water supply system throughout the experiment. For the systems of MFC-extV, an external voltage maintained at 25–50 mV was applied to the MFC circuit by a voltage stabilizer (AD-8735A; AND Company Ltd.) throughout the period of measurements.

4.2.2 Data analysis

Eh distribution patterns in soils between the treatments were compared with depths and submerged periods. In addition, N_2O flux rates of the primary study is shown as supportive data, as for the basis of current study. All statistical analyses described in this article were performed using IBM SPSS Statistics version 24.

4.3 Results and Discussion

4.3.1 Eh distributions with time and soil depth

Soil Eh distributions at depth profiles with period of submergence is shown for each treatment by **figure 4.2**. The soils at 10 cm and 20 cm were comparatively of higher Eh values than that of 30 cm, especially with MFCs and are followed by MFC-extV. Non-MFCs didn't show much variations between oxidizing and reducing conditions with depth profiles, proving that more frequently they had reducing conditions. MFC-extV showed much variations of Eh with time and depths.

Univariate analysis of Variance was conducted for studying the variations of Eh between treatments and soil depth profiles. The soil redox data has significant difference with treatments as well as depth profiles, and also there are significant interaction effects between depths and treatments, at p=0.05 level. However, MFC-extV treatment is less influencing on controlling redox potentials of soils, unlike that is of MFCs. Further, the redox distribution profiles were compared with relative to soil portions (denoted as middle, left and right) at each depth, especially with MFCs and MFC-extV treatments as shown in **Figure 4.3**. External voltage applied soil portions showed Eh changes in an alternative pattern in soils between and near electrodes. Adjacent soil portions had opposite charges even at the same depth profiles which could be denoted as + Δ Eh and –Δ Eh. At 10 cm depths, right side soil portion was positively charged frequently, while at 30 cm depth, left side portion was positively charged. Middle soil portions at all 3 depths, which were negatively charged frequently, were held by the attractions of those positively charged soil particles on its adjacent sides. However, it is not certain of reasons for such random distributions, the differently charged particles could be due to the electrolysis chemical reactions in the soils occurred non-spontaneously due to the DC supply of voltage. Other than microbial

fuel cell, the systems with external voltage, could partially performed as an electrolytic cell, causing non-spontaneous reducing reactions.

Therefore, negatively charged soil zones could have formed randomly as a result of the external voltage applied. These reducing reactions would be causative of not more electrons being transferred to the anode from the whole soil.

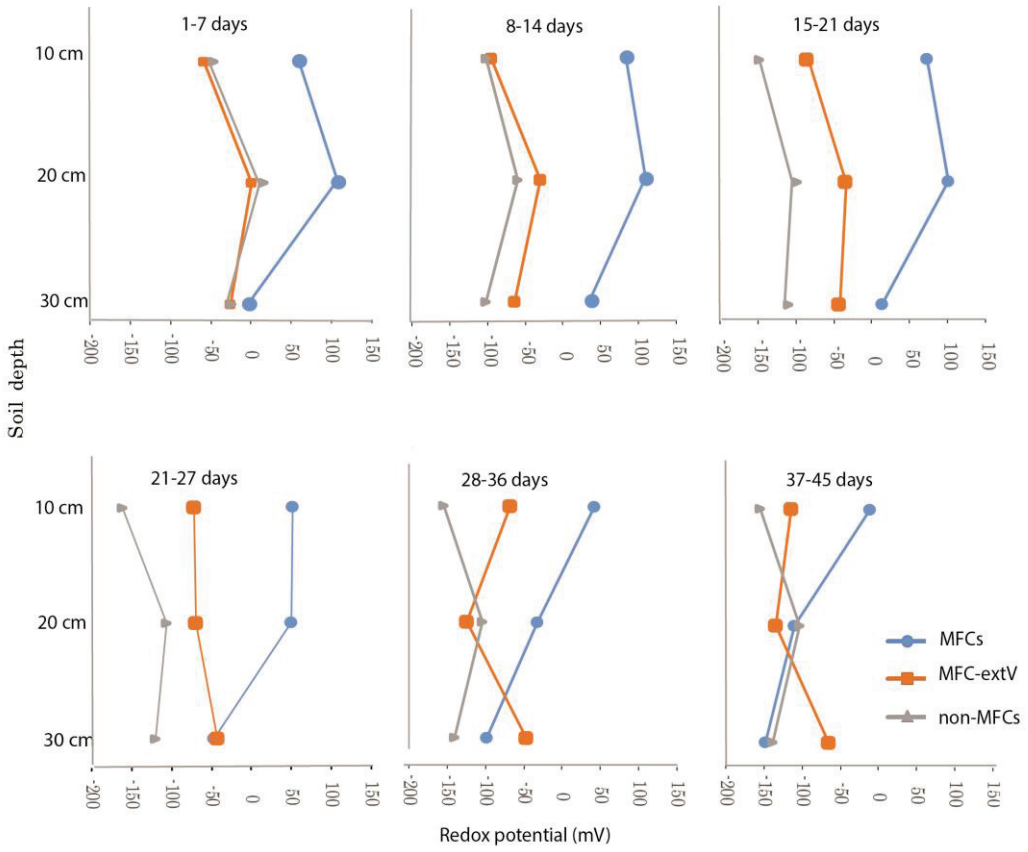


Figure 4.2: Soil Eh - depth profiles with period of submergence. Mean ±SD of Eh over the whole period are:
MFCs (52 ± 46), MFC-extV (-81 ± 97) at 10 cm.
MFCs (39 ± 82), MFC-extV (-64 ± 85) at 20 cm
MFCs (-39 ± 100), MFC-extV (-47 ± 143) at 30 cm

Also, distribution of the voltage gradients in the soil between the two electrodes might have complex patterns. The voltage gradients may vary from point to point between the two electrodes, and are not evenly distributed. They should be steep near the anodes, and spread-out in the middle between them. Near the electrodes where the current enters or leaves the soil could have voltage gradients become stronger. In the other area between the two electrodes, the current becomes diffused to result the voltage gradients also be diffused. Thus, the transfer of electrons towards the anode for current generation was not totally covered by whole soil, but that was limited near the area of anode.

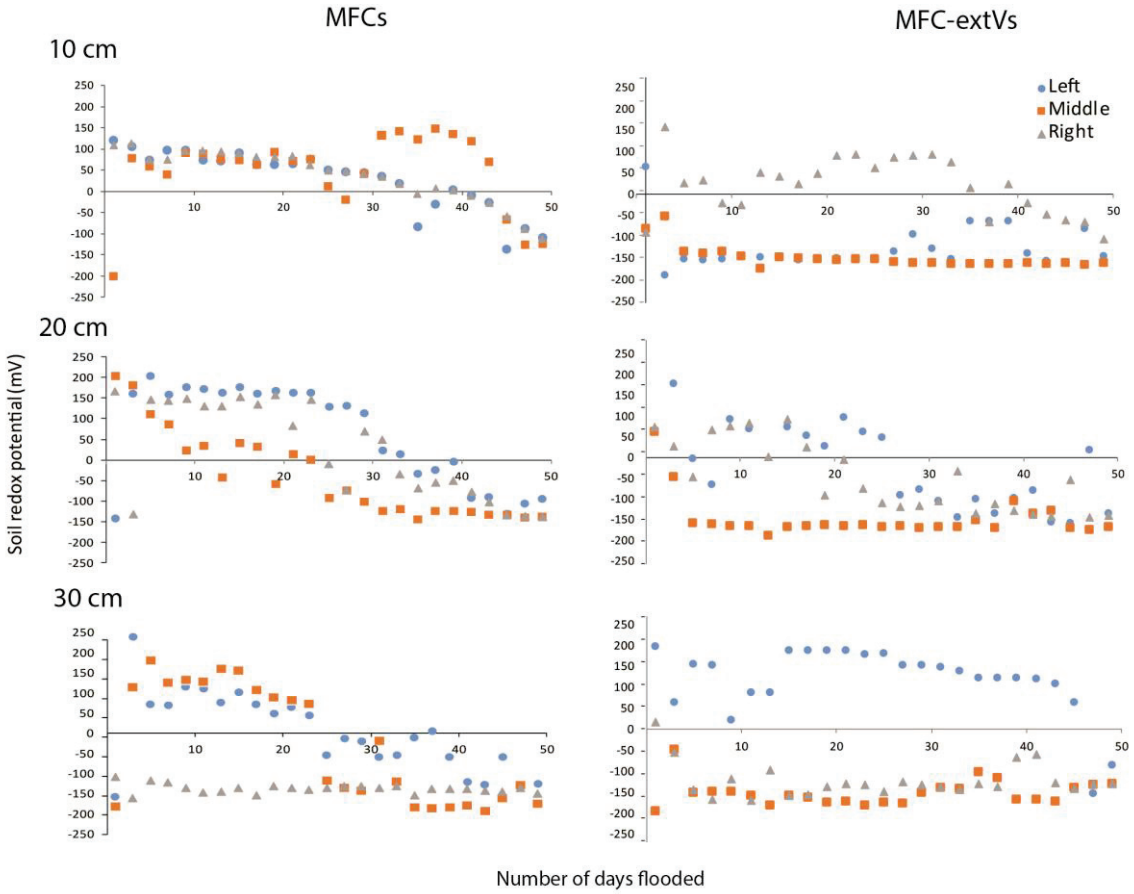


Figure 4.3: Soil Eh distributions with depth layers
(Eh measured at left, middle and right side portions of each depth level)

It is clear that the externally applied voltage was not in help with the efficiency of MFC-extV treatments, due to the voltage gradients and formation of soil zones that held together by electric charges. Thus, soil zoning could also be a barrier for free movement of electrons to the anode. Overall, effects of MFC-extV treatments on soil redox control were lower compared to that of MFCs treatments, but were higher compared to that of non-MFCs.

4.3.2 N_2O flux with MFC applied soils

The time series of N_2O flux of each treatment is shown in **Figure 3.5** in chapter 3. Both MFC and MFC-extV treatments showed lower flux rates than that of non-MFCs. While N_2O flux showed a significant difference between MFCs and non-MFCs, there was no statistically significant difference between MFCs and MFC-extV. This was in accordance with multiple comparisons as shown in **figure 3.6** in chapter 3. As a consequence of relatively high redox potential in MFCs and MFC-extV, denitrification processes were suppressed, and as a result N_2O flux was also suppressed. From the experiment data of primary study, we investigated that MFC systems have the potential to control the denitrification process. But, it was not clear how externally applied voltage effected on its efficiency.

4.4 Conclusion

MFCs treatments had Eh controlling effects both spatially and temporary. But, with that of MFC-extVs treatments, Eh controlling was not much effective as of MFCs. One possible reason could be due to the formation of charged soil zones that held adjacently together by their net electric charges. These random effects could resulted through electrolysis chemical reactions, most of which are non-spontaneous reducing reactions in soils due to the externally applied DC voltage. The other reason could be due to the formation of voltage gradients, steeper at the electrodes and diffused away from them. Thus, most electrons in whole soils of MFC-extV treatments were not transferred freely to the anode, but were from near anode area where the external voltage was connected through. As a consequence of relatively high redox potential in MFCs treatments, denitrification processes were suppressed, and as a result N_2O flux was also suppressed, compared to that of MFC-extV treatments.

CHAPTER 5

QUANTIFICATION OF DENITRIFYING BACTERIA WITH RESPONSE TO MICROBIAL FUEL CELL APPLICATIONS IN PADDY SOILS

5.1 Introduction

A quantification study of denitrifying bacteria was conducted for further understanding of denitrification rates with respect to MFC treatments and controls. The study was also based on our primary study of nitrogen behaviour in flooded rice soils, under three conditions: MFC systems, MFC systems with an externally applied voltage, and non-MFC systems as controls. The MFC systems with an externally applied voltage in the previous study were aimed at increasing the efficiency of MFCs, expectedly with a rapid conveying of electrons towards the anodic area. In the rest of this article, we refer to the MFC systems as MFCs, MFC systems with an externally applied voltage as MFC-extV, and non-MFC systems as non-MFCs. The aim of this study is to compare the amounts of denitrifying bacteria in MFC applied rice soils and controls with relative to one of their respective nitrite reductase genes - nirS.

5.2 Material and methods

5.2.1 Soil DNA extractions

Soil microbial DNA was extracted from 0.25 g of soil, by using Power soil® DNA isolation kit (MO BIO laboratories, Inc.) according to the manufacturer's instructions. The extracts were stored at -21 °C until analysis by polymerase chain reaction (PCR). For each treatment, three replicates were analyzed at a time.

5.2.2 PCR amplification

Primers used for amplification of nirS target gene fragment are nirS4F (5' TTC RTC AAG ACS CAY CCG AA 3') and nirS6R (5' CGT TGA ACT TRC CGG T 3') according to Throback *et al* (2004). PCR was conducted to amplify nirS gene which produces the desired fragment of 336 bp approximately. PCR amplification was performed in a total volume of 25 µl containing, 2.5 µl of 10x PCR buffer (Mg^{2+} free, Takara), 15 mM $MgCl_2$, 200 µM of deoxy nucleotide triphosphate (DNTP), 1.25 U of Taq polymerase (Takara), 1 µM of each primer. Reaction conditions used for amplification are with an initial denaturation of the DNA at 94 °C for 2 min, followed by 35 cycles of at 94 °C for 30 s, at 57°C for 1 min and at 72°C for 1 min. The reaction was completed at 72°C

after 10 min. The reaction mixtures were place in a mini-cycler (LifePro, Bioer Technology Co.,Ltd) for amplification. The size of PCR products obtained from nirS gene amplifications were confirmed by 2% agarose gel run at 100 V for 23 minutes. Gel was stained with Ethidium Bromide for 20 minutes and visualized by using the Gel Doc 2000 system (BIO-RAD, USA).

5.2.3 Cloning, sequencing, Quantitative PCR Calibration curves

Gel bands of the PCR amplicons of expected size were excised, visualizing through a benchtop UV transilluminator. Excised gel DNA was purified by using a gel extraction kit (QIAquick Gel extraction kit, Qiagen), after which they were ligated into pMD19-T cloning vector (Takara bio Inc.), and transformed into E-coli DH5α cells (Takara) by using the DNA ligation kit (Mighty Mix,Cat.#6023) according to the manufacturer's instructions. Blue-white screening technique was used to identify colonies for subsequent analysis. By spreading 100 µl of the transformation culture on agar plates, containing solutions (filtered through 0.25 µm) of ampicillin (50 µg/ml), 100 µl of X-gal (20 mg/ml), and 100 µl of IPTG (100mM), and then incubated (Incubator IC600) overnight at 37 °C. Transformants appeared in white colonies, which indicated the insertion of the PCR product, were randomly collected and verified the presence of appropriate insert in the recombinant plasmids by performing a colony PCR. The primers used were the vector primers, M13 forward (5' GTA AAA CGA CGG CCA G) and M14 Reverse (5' CAG GAA ACA GCT ATG AC 3'). The reaction was totally in a 25 µl mixture, containing 2.5 µl of 10x Taq buffer,2µl of DNTP (2.5 mM), 1µl each M13 primer (10µM), 0.125 µl of Ex-Taq polymerase(5U/µl), plasmid DNA 1µl(≈20ng/µl) with remaining accounted by PCR-grade H_2O 17.375µl. Thermal cycling conditions of the colony PCR were with an initial denaturation of the DNA at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, at 50°C for 30 s and at 72°C for 30 s. The reaction was completed at 74°C after 8 min. The sizes of colony PCR products were confirmed by 2% gel electrophoresis, and the remaining reaction mixture was purified by using ExoSAP –IT^{TM} reagent according to the manufacturer's instructions. Purified PCR product (9µl) with each nirS4F and nirS6R primers (1µl) separately was used as the template DNA of positive transformants which was sent to the Genomic Center of Gifu University for sequencing. The gene sequence was used for BLAST searches of the

GeneBank database. After this confirmation step, a single white colony containing recombinant plasmids was inoculated into 200 µl LB broth culture medium with ampicillin (50 µg/ml), and incubated at 37 °C for an overnight. Recombinant plasmid DNA was extracted by using mini-prep kit (QIAprep miniprep, Qiagen) according to the manufacturer's instructions. Concentrations of purified recombinant plasmid DNA was measured by using Quantifluor dsDNA system (Promega, USA). Standards were prepared with serial dilutions of plasmids containing between 10^8 and 10^2 nirS copies, calculated per µl according to the equation (1) from the initial concentration of recombinant plasmids extracted. The known plasmid concentrations were plotted as the log of initial target DNA copy numbers versus threshold cycle (Ct) value. The amplification efficiency (E) was estimated using the slope of the standard curve. They were replicated for higher regression co-efficient R^2 value and amplification efficiency (E) exceeding 0.99.

$$\text{Copy number}/\mu l = (ng \times 6.022 \times 10^{23}) / (length \times 1 \times 10^9 \times 650) \quad (1)$$

Where 6.022×10^{23} = Avogadro's number, average weight of one base pair = 650, and measured concentrations of initial plasmid DNA in ng per µl and total length of the recombinant plasmid gene (bp) can be substituted relevantly.

5.2.4 Quantification by Real-time PCR

Real-time PCR was performed using a Real-time PCR System (Thermal Cycler dice, TP800, Takara). Reaction was in a total volume of 25µl containing, 12.5 µl of SYBR Premix Ex Taq (Takara), 1µl of each primer nirS4F and nirS6F (10 µM) and 1µl of template DNA (DNA sample or cloned DNA for standard curves). Thermal cycles used for amplification are with an initial denaturation of the DNA at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 57°C and 1 min at 72°C. After each reaction was completed, a melting curve was generated to confirm the specificity of each PCR assay (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s). All measurements were of three replicates.

5.3 Data analysis

5.3.1 Statistical analysis

Using daily averaged redox potential and N_2O flux observed from primary study, difference between the treatments were compared. In addition, by conducting statistical analysis such as one-way ANOVA and multiple comparison, differences between treatments were statistically examined for redox, N_2O flux and nirS gene copy numbers. All statistical analyses described in this article were performed using IBM SPSS Statistics version 24.

5.4 Results and Discussion

5.4.1 Real-time PCR analysis of nirS genes in soil

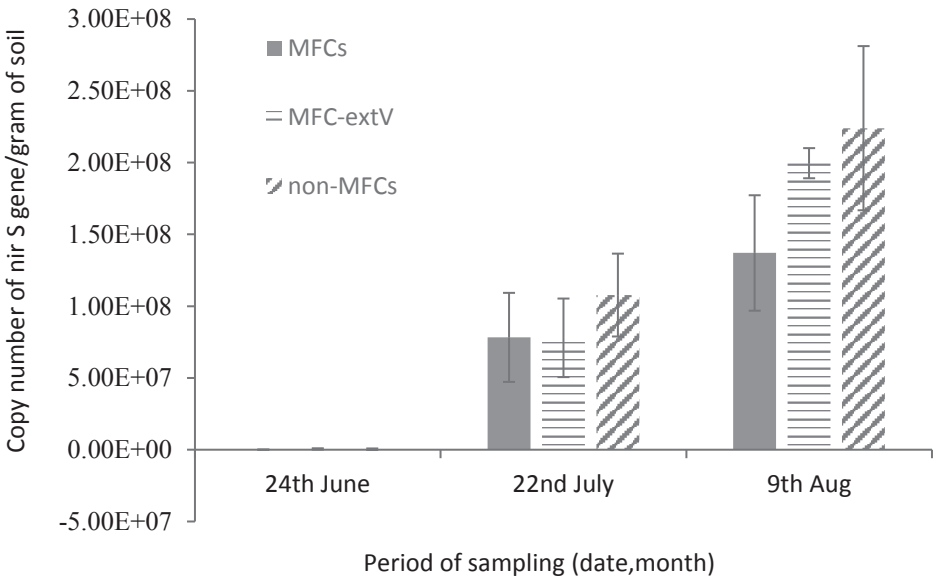


Figure 5.1 Mean± SD of nirS gene targeted cells with treatments and controls over the period. Error bars represent the Standard deviation.

nirS gene was increased with all systems over the period, proving that denitrifying conditions prevailed over the time under the flooded conditions, but with different rates with treatments and controls. Mean nirS gene cell numbers were different with treatments and periods as shown by **figure 5.1**. In the sampling event of August, significant differences were found between MFCs and

non-MFCs (p=0.002). The greater abundance of nirS gene copy numbers related to non-MFC controls were during the end of the reproductive stage of rice. The significant difference ($p < 0.05$ level) showed that MFCs treatments were of the lower denitrifying capabilities than that were of non-MFCs controls. The result implies that during vegetative and reproductive stages of rice, nirS gene was not much effectively controlled by MFCs. The higher nirS copy numbers in non-MFCs on 9th August coincide with its higher N_2O flux and lower redox potentials as well, compared to that of MFCs. The trends further implies that MFCs and MFC-extV treatments had almost the same denitrifying capabilities, when they were compared to non-MFC treatments throughout the experimental period. However, nirS gene could only partially interpret the capability of denitrification. Also, robust patterns and conclusions could not be obtained from limiting to the analysis of nirS genes, whereas the other functional genes including nirK are also important to make a conclusion, since their functions are independent.

Figure 5.2 shows the standard curve of log number of initial target gene copy numbers against the threshold cycle (Ct) value. Regression co-efficient (R^2 value) and amplification efficiency (E) of the standard calibration curve were 0.999 and 105.9 respectively.

Figure 5.3 shows the melting curve analysis of real-time PCR quantification analysis. Melting point analysis was by gradually increasing temperature from 60 to 95°C, continually measuring fluorescence for each sample.

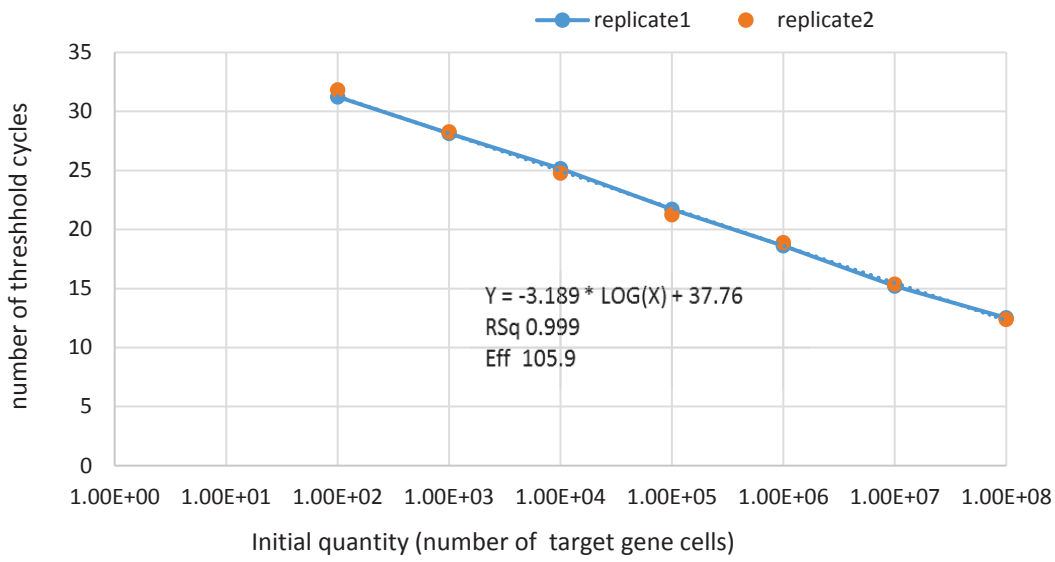


Figure 5.2 Standard curve of nirS gene targeted cells.

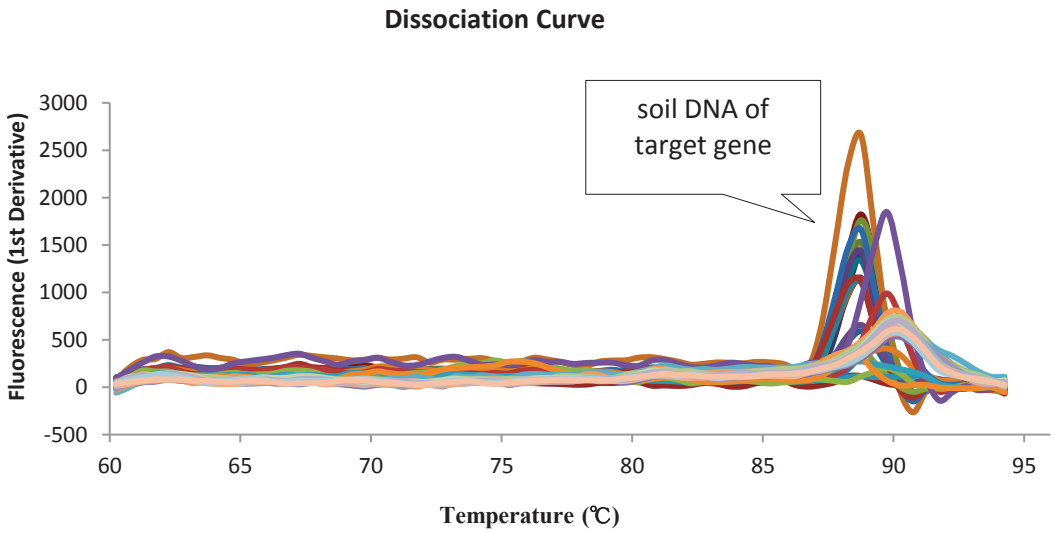


Figure 5.3 Dissociation curve of real-time PCR analysis

5.4.2 Gas flux and Redox changes

Overlay of mean N_2O flux and redox potentials with relevant to the rice growth stages, shown by **figure 3.7** in chapter 3 could be compared with trends of nirS gene copy numbers observed in June 24th (start of vegetative stage), July 22nd (start of reproductive stage) and August 9th (start of maturation stage). MFCs and MFC-extV systems were not significantly differ with flux and redox conditions, but the significant differences were found between MFCs and non-MFC control systems

(one-way ANOVA with multiple comparisons of HSD Turkey test, $p < 0.05$). However, these significant differences were also shown to be at the end of reproductive stage through the start of maturation stage as shown by **figure 3.5** in chapter 3. Simultaneously, the trends of denitrifying nirS genes copy numbers of treatments and controls are also consistent with that of soil redox potentials as shown by **figure 3.3** in chapter 3. MFCs and MFC-extV treatments followed approximately the same trends of higher redox potentials, while non-MFC controls followed the lower redox potential trends. Therefore, observed trends of nirS gene copy numbers, N_2O flux and redox potentials are consistent together with respective to each treatment and control systems.

5.4.3 C/N ratios in soil

C/N ratio is an important indicator of electron availability, and of denitrification potentials as well. Trends of C/N ratios in MFC treatments and controls are shown by **figure 3.8** in chapter3. In terms of C/N ratio, we could not clearly elaborate the significant difference in nirS gene copy numbers related to 9th August between non-MFCs and MFCs. However, the trends of nirS gene copy numbers related to the samplings in June and July are supportive with C/N ratios trends relevantly.

5.5 Conclusion

Observed trends of nirS gene quantities, N_2O flux and redox potentials are consistent together with respective to each treatment and control systems. The higher nirS gene quantities coincide with higher N_2O flux and lower redox potentials of non-MFCs, compared to that of MFCs. The trends further implies that MFCs and MFC-extV treatments had almost the same denitrifying capabilities, when they were compared to that of non-MFC treatments throughout the experimental period. The trends of nirS gene quantities related to the samplings in June and July were only supportive with C/N ratios trends relevantly. Through nirS gene analysis, the extent of denitrification could only partially be interpreted. However, robust patterns and conclusions could be obtained from the analysis of nirS genes with the other functional genes.

CHAPTER 6

SUMMARY & CONCLUSIONS

6.1 Summary & Conclusions

- We attempted to confirm the applicability of MFC theory for suppressing denitrification in flooded rice soils.
- With relatively high redox potential in MFCs treatments, denitrification processes were comparatively suppressed. As a result, N_2O flux was also suppressed in MFCs, compared to that of MFC-extV treatments and non-MFCs controls.
- Trends of soil redox potential, nirS gene quantities and the C/N ratios in MFCs and MFC-extV systems were also consistent with this result.
- Electrons mostly utilized by MFC-extV circulation is of near anode, but not equally from whole soils, which might be due to the voltage gradients and soil zoning with electrolysis reactions. Therefore, the MFCs plus external voltage had no additional controlling effect as expected.
- Trends of nirS gene quantities further implies that MFCs and MFC-extV treatments had almost the same lower denitrifying capabilities, when they were compared to non-MFC treatments throughout the experimental period. Also, the higher nirS cell numbers coincide with higher N_2O flux and lower redox potentials of non-MFCs, compared to that of MFCs.
- In conclusion, we confirmed the applicability of MFC to control soil redox potential and thereby suppress denitrification nitrogen losses in flooded rice fields.

REFERENCES

1. Akiyama, H., Yagi, K., and Yan, X. (2005). Direct N_2O emissions from rice fields: Summary of available data. Global Bio Geo Chemical Cycles 19, GB 1005
2. Arends, B. A., Speeckaert, J., Blondeel, E., Jo De Vrieze, Boeckx, P., Verstrate, W., Rabaey, K., and Boon, N. (2014). Greenhouse gas emissions from rice microcosms amended with a plant microbial fuel cell, Applied Microbiology and Biotechnology. 98: 3205-3217
3. Arends B. A., Blondeel E., Tennison S. R., Boon, N. and Verstrate, W. (2012). Suitability of granular carbon as an anode material for sediment microbial fuel cells. Journal of Soil Sediments.12:1197-1206
4. Arends, B. A., and Verstrate, W. (2012). Minireview of 100 years of microbial electricity production: three concepts for the future. Microbial Biotechnology. 5(3): 333-346
5. Ashley E. F. and Nevin, K. P. (2010) Microbial fuel cells. A current Review. Energies. 3: 899-919
6. Aulakh, M. S., Wassmann, R.., Bueno, C., Kreuzwieser, J., and Rennenberg, H. (2001). Characterization of Root Exudates at Different Growth Stages of Ten Rice (Oryza sativa L.) Cultivars. Plant Biology. 3 : 139 -148
7. Beauchamp, E.G, Trevors, J.T. and Paul, J.W. (1989). Carbon Sources for Bacterial Denitrification. In: Stewart B.A. (eds) Advances in Soil Science. Advances in Soil Science, vol 10. Springer, New York
8. Betlach, M. R., and Leeuwenhoek, A.V., (1982). Evolution of bacterial denitrification and denitrifier diversity. 48(6) : 585-607
9. Bond, D. R. and Lovely, D. R. (2002). Electricity production by Geobacter sulfurreducens attached to electrodes. Applied and Environmental Microbiology. 69(3) : 1548:1555
10. Braker, G., and Tiedje, J. M. (2003). Nitric oxide reductase (norB) genes from pure cultures and environmental samples. Applied and Environmental Microbiology. 69:3476-3483

11. Cabello, P., Roldan, M. D., and Vivian, C. M. (2004). Nitrate reduction and the nitrogen cycle in archaea. Microbiology. 150:3527-3546
12. Cardenas, L. M., Hawkins, J. M. B., Chadwick, D., and Scholefield, D. (2003). Biogenic gas emissions from soils measured using a new automated laboratory incubation system, Soil Biology and Biochemistry. 35: 867–870
13. Crutzen, P. J. (1970). The influence of nitrogen oxides on the atmosphere ozone content. Quarterly Journal of the Royal Meteorological Society. 96: 320-325
14. Dandie, C. E., Miller, M. N., Burton, D. L., Zebarth, B.J., Trevors, J.T., and Goyer, C. (2007). Nitric oxide reductase – targeted real-time PCR quantification of denitrifier populations in soil. Applied and Environmental Microbiology. 73: 4250-4258
15. Derek R. Lovley (2010). Powering microbes with electricity: direct electron transfer from electrode to microbes. Society for applied microbiology and Blackwell publishing Ltd. Environmental microbiology Reports. 3: 27-35
16. Felber, R., Conen, F., Flechard, C. R., and Neftel, A. (2012). The acetylene inhibition technique to determine total denitrification (N_2 +N_2O) losses from soil samples: potentials and limitations. Biogeosciences discussions. 9: 2851–2882
17. Felber, R. Conen, F., Flechard, C. R., and Neftel, A. (2012). Theoretical and practical limitations of the acetylene inhibition technique to determine total denitrification losses. Biogeosciences.9:4125-4138
18. Ghosh, S., Majumdar, D., and Jain, M. C. (2003). Methane and nitrous oxide emissions from an irrigated rice of North India, Chemosphere 51: 181-195
19. Groffman, P. M., Altabet, M. A., Bohlke, J. K., Butterbach-Bahl, K., David, M. B., Firestone, M. K., Giblin, A. E., Kana, T. M., Nielsen, L. P., and Voytek, M. A. (2006). Methods for measuring denitrification, Diverse approaches to a difficult problem, Ecological Applications. 16: 2091–2122
20. Groffman, P. M., and Robertson, G. P. (2015). Nitrogen transformations. Soil microbiology, ecology and biochemistry. Fourth edition. Academic Press, Burlington, Massachusetts, USA. 421-446

21. Gruber, N. and Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle, Nature, 451/17

22. Henry, S., Baudoin, E., Lopez-Gutierrez J.C., Martin-Laurent, F., Brauman, A. and Phillipot, L. (2004). Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. Journal of microbiological methods. 59: 327-335

23. Henry, S., Bru, D., Stres, B., Hallet, S., Phillipot, L. (2006). Quantitative detection of the nosZ gene encording nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Applied Environmental Microbiology. 72: 5181-5189

24. Hou A.X., Chen, G.X., Wang, Z.P., Van, Cleemput, O. and Patrick, W.H. (2000). Methane and nitrous oxide emissions from a rice field in relation to soil redox and microbiological processes. Soil Science Society of America. 64: 2180-2186

25. Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., Vander Linden, P. J., Dai, X., Maskell, K., and Johnson, C. A. (2001). Climate change 2001: The scientific basis contribution of working group 1 to the third assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, United Kingdom

26. Huang, S., Pant, H. K., and Lu, J. (2007). Effects of water regimes on nitrous oxide emissions. Ecological engineering. 31: 9-15

27. Husson, O. (2013). Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: A transdisciplinary overview pointing to integrative opportunities for agronomy, Plant and Soil, 362, pp.389-417.

28. Iida, T., Deb, S. K., and Kharbuja, R. M. (2007). Nitrous oxide emission measurement with acetylene inhibition method in paddy fields under flood conditions. Paddy and Water Environment. 5: 83-91

29. Ishii, S., Ikeda, S. Minasawa, K., and Senoo, K. (2011). Nitrogen Cycling in Rice Paddy Environments: Past Achievements and Future Challenges: Minireview. Microbes and Environments. Vol. 26(4): 282–292

30. Ishii, S., Yamamoto, M., Kikuchi, M., Oshima, K., Hattoru, M., Otsuka, S. and Senoo, K. (2009). Microbial populations responsive to denitrification-inducing conditions in rice paddy soil as revealed by comparative 16srRNA gene analysis. Applied and environmental microbiology, American society for Microbiology.75: 22

31. Jury, W. A., Letey, J., and Collins, T. (1982). Analysis of chamber methods used for measuring nitrous oxide production in the field. Soil Science Society of America Journal. 46.2: 250-256.

32. Khalil, K,. Mary, B. and Renault, P. (2004). Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O_2 concentration. Soil Biology and Biochemistry. 36: 687-699

33. Kaewpradit, W., Toomsan, B., Vityakon, P., Limpinuntana,V., Saenjan, P., Jogloy, S., Patanothai, A., and Cadisch, G. (2008). Regulating mineral N release and greenhouse gas emissions by mixing groundnut residues and rice straw under field conditions. European Journal of Soil Science. 59: 640-652

34. Kaku, N., Yonezawa, N., Kodama, Y., and Watanabe, K. Plant/microbe cooperation for electricity generation in a rice paddy field. Applied Microbiology and Biotechnology (2008). 79:43-49

35. Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., and Philliot, L. (2006). Abundance of narG, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. Applied Environmental Microbiology.72: 5957-5962

36. Keeny, D. R., and Sahrawat, K. L. (1986). Nitrogen formations in flooded rice soils, Fertilizer Research. 9: 15- 38

37. Kouzuma, A., Kaku, N., and Watanabe, K. (2014). Microbial electricity generation in rice paddy fields: recent advances and perspectives in rhizosphere microbial fuel cells, Applied Microbiology and Biotechnology. 98: 9521-9526

38. Krishna Mohan, T.V., Nancharaiah, Y.V., Venugopalan, V.P., and Satya Sai, P.M. (2016). Effect of C/N ratio on denitrification of high-strength nitrate wastewater in anoxic granular sludge sequencing batch reactors, Ecological Engineering, 91: 441–448

39. Lambers, H. (2013). Redox potential (Eh) and pH as drivers of soil, plant/microorganism systems: a transdisciplinary overview pointing to integrative opportunities for agronomy, 362(1–2), pp. 389–417.

40. Lan, T., Han, Y., Roelcke, M., Nieder, R. and Cai, Z. (2014). Sources of nitrous and nitric oxides in paddy soils: Nitrification and denitrification. Journal of environmental sciences. 26 : 581-592

41. Liu, J., Hou, H., Sheng, R., Chen, Z., Zhu, Y., Qin, H. and Wei, W. (2012). Denitrifying communities differentially respond to flooding drying cycles in paddy soils. Applied Soil Ecology. 62 : 155-162

42. Lovley, D. R. (2006). Bug Juice: harvesting electricity with microorganisms. Nature Reviews. 4: 497-508

43. Lovley, D. R. (2006). Microbial fuel cells: novel microbial physiologies and engineering approaches. Current Opinion in Biotechnology, 17: 327–332

44. Lovley, D. R. (2008). The microbe electric: conversion of organic matter to electricity. Current Opinion in Biotechnology, 19: 564–571

45. Lovley, D.R. (2010). Powering microbes with electricity: direct electron transfer from electrode to microbes. Society for applied microbiology and Blackwell publishing Ltd. Environmental microbiology Reports, 3: 27-35

46. Lovley, D.R. (2011). Powering microbes with electricity: direct electron transfer from electrodes to microbes. Environmental Microbiology reports. 3(1) : 27-35

47. Lovley, D.R. and Nevin, K.P. (2011). A shift in the current: New applications and concepts for microbe-electrode electron exchange. Current Opinion in Biotechnology, 22:441–448

48. Minami, K. (1987). Emission of nitrous oxide from agro-eco system. Japan Agricultural Research Quarterly. 21(1): 22-27

49. Mosier, A. R. (1980). Acetylene inhibition of ammonium oxidation in soil. Soil Biology and Biochemistry. 12: 443-444

50. Mosier, A. R., Duxbury, J. M., Freney, J. R., Heinemeyer, O., and Minami, K. (1996). Nitrous oxide emissions from agricultural fields: Assessment, measurement and mitigation. Plant and Soil. 181: 95-108

51. Olson, R. A., and Halstead, E. H. (1974). The fertilizer nitrogen problem. Bulletin of International Atomic Energy Agency (16). In AGRIS (since 2012). v26-29(5)

52. Parkin, T. B., Kaspar, H. F., Sexstone, A., and Tiedje, J. M. (1984). A gas flow soil core method to measure field denitrification rates. Soil Biology and Biochemistry. 16: 323-330

53. Pathak, B.K., Iida, T., and Kazama, F. (2007). Denitrification as a component of nitrogen budget in a tropical paddy field. Global NEST Journal, 9 :2, 159-165

54. Philippot, L. (2005). Tracking nitrate reducers and denitrifiers in the environment. Biochemical Society Transactions. 33: 200-204

55. Philliot, L., Hallin, S., and Schloter, M. (2007). Ecology of denitrifying prokaryotes in agricultural soil. Advanced Agronomy. 96: 246-305

56. Ponnamperuma F. N. (1972). The chemistry of submerged soils. Advanced Agronomy. 24 : 29-96

57. Pramanik, P., Hagu, M. W., Kim, S. Y., and Kim, P. J. (2014). Carbon and nitrogen accumulation in soil aggregates determine nitrous oxide emissions from cover crop treated rice paddy soils during fallow season. The Science of the Total Environment. 490: 622-628

58. Rabaey, K. and Verstraete, W. (2005). Microbial fuel cells: novel biotechnology for energy generation trends in Biotechnology. 23-6

59. Randeniya, L. K., Vohralik, P. F. and Plumb, I. C. (2002). Stratospheric ozone depletion at northern mid latitudes in the 21st century: the importance of future concentrations of greenhouse gases nitrous oxide and methane. Geophysical Research Letters. 29(4)

60. Reddy, K. R., and Patrick, W. H. (1986). Denitrification losses in flooded rice fields, Fertilizer Research. 9: 99-116

61. Reddy, K. R., Patrick, W. H. and Lindau, C. W. (1989). Nitrification-denitrification at the plant root-sediment interface in wetlands, American Society of Limnology and Oceanography. 34(6): 1004-1013

62. Schamphelaire, L., Bossche, L.V., Dang, H.S., Hofte, M., Boon, N., Rabaey, K. and Verstrate, W. (2008). Microbial fuel cells generating electricity from rhizodeposits of rice plants. Environmental Science and Technology, 42 : 3053-3058

63. Shan, Y., Cai, Z., Han, Y., Johnson, S.E. and Buresh, R.J. (2008). Organic acid accumulation under flooded soil conditions in relation to the incorporation of wheat and rice straw with different C: N ratios. Soil Science & Plant Nutrition. 54: 46-56

64. Snakin, V.V., Prisyazhnaya A.A. and Kovacs-Lang, E. (2001). Soil liquid phase composition. Elsevier Science B.V, Amsterdam

65. Takenezawa, K., Nishio, K., Kato, S., Hashimoto, K., and Watanabe, K. (2010). Factors Affecting Electric Output from Rice-Paddy Microbial Fuel Cells. Bioscience, Biotechnology, and Biochemistry, 74:6, 1271-1273

66. Throback, I. N., Enwall, K., Jarvis, A. and Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE, FEMS Microbiology Ecology (Amsterdam).49: 401-417

67. Tiago, Z., Bayer, C., Zanatta, J.A., Vieira, F.C.B. and Anghinoni, I. (2011). Mitigation of methane and nitrous oxide emissions from flood-irrigated rice by no incorporation of winter crop residues into the soil. Brazilian Journal of Soil Science (R. Bras. Ci. Solo).35: 623-634

68. Tiedje, J. M., Simkins, S. and Groffman, P. M. (1989). Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods. Plant and Soil. 115: 261–284

69. Wang, Z., Delaune, R. D., Masscheleyn, P. H. and Patrick, W. H. (1993). Soil redox and pH effects on methane production in a flooded rice soil.Soil Science Society of America Journal. 57: 382-385

70. Wrage, N., Velthof, G. L., Beusichem, M. L. Van., Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. Soil Biology and Biochemistry 33: 1723-1732

71. Xiong, Z. Q , Xie, Y. X, Xing, G. X., Zhu, Z. L. and Butenhoff, C. (2006). Measurements of Nitrous Oxide Emissions from Vegetable Production in China. Atmospheric Environment. 40(12): 2225–2234

72. Zhu, Y. G. (2014). Key microbial processes in nitrous oxide emissions of agricultural soil and mitigation strategies, Huan Jing Ke Xue (China). 35(2): 792-800

73. Zumft, W. G. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxide type. Journal of Inorganic Biochemistry. 99: 194-215

APPENDIX 1

Several experimental events and used equipment during the research

a. A gas sampling event during the assessments of denitrification process with MFC treatments and controls (Primary study, summer, 2016)

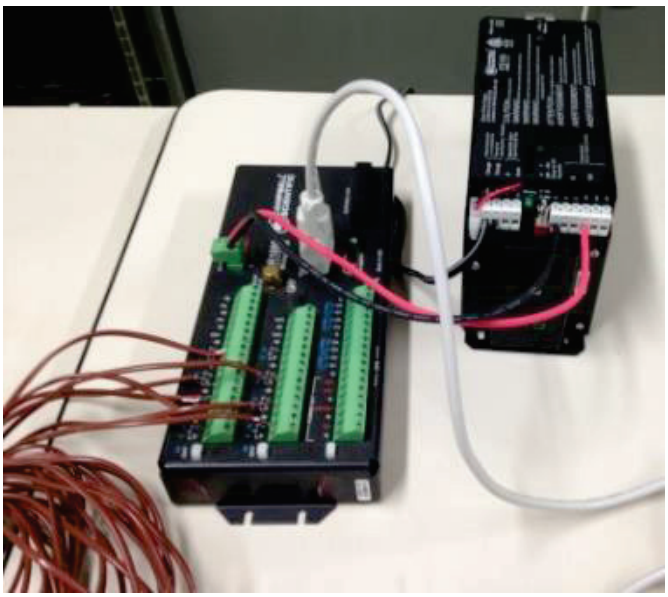
a.

b.

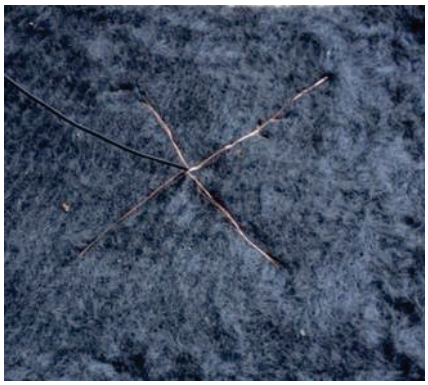
b. An instance of soil redox monitoring (summer, 2016)

c.

c. An instance of MFC voltage output (summer, 2016)



Data logger CR-1000; Campbell Scientific Inc.

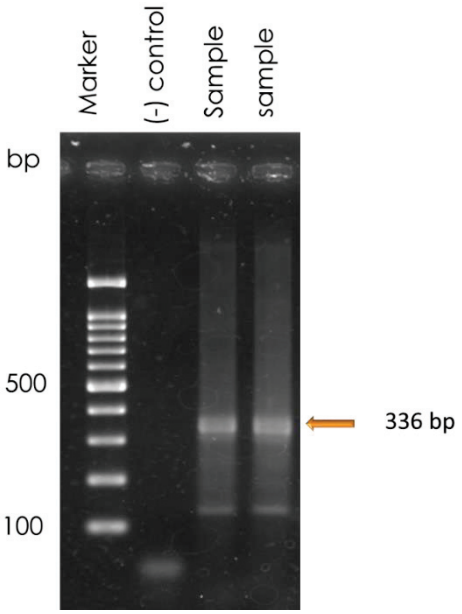


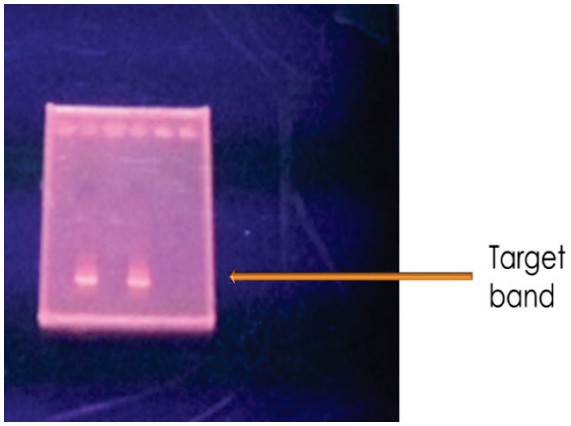
MFC anode – Carbon mat

Eh meters (PRN-41; Fujiwara Factory Co., Ltd.)

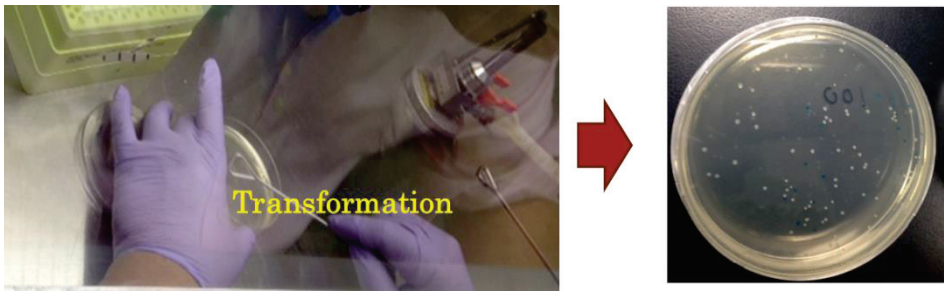
APPENDIX 2

Main steps during the preparation of standard DNA for qPCR analysis

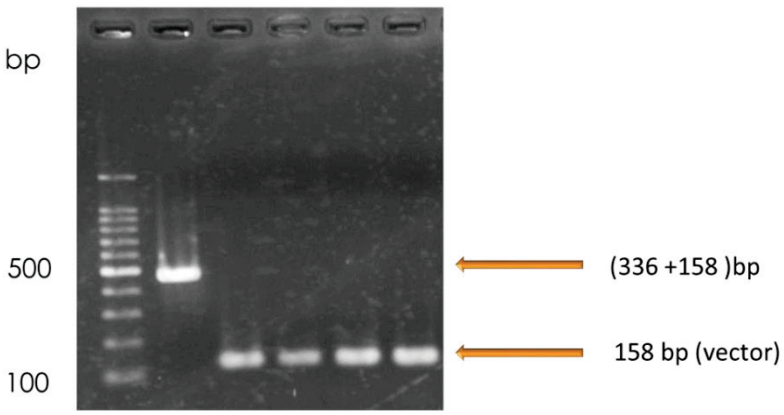




PCR amplification with nir S primers **DNA extraction from gel**



TA Cloning (with T-vector pMD19 and Ligation kit (Mighty Mix) and transformation into competent cells of Ecoli DH5α)



Confirmation of insert DNA with Colony PCR (M13primer M4, M13primer RV)

APPENDIX 3

Conversions of (volume/volume) gas concentrations into (mass/volume) during gas flux analysis

Volume to volume concentrations were converted to mass/volume concentrations using the formula as given in the example below.

X ppm (volume/volume) N_2O converting into g/m^{-3} (mass/volume)

E.g., When sample is X ppm,

X m^3 of N_2O is in $\mathbf{10^6}$ m^3 of air mixture, hence $\mathbf{10^{-6}}$ **X** of N_2O is in $\mathbf{1m^3}$ of air mixture.

If sample has N_2O of X ppm at 25 degrees of Celsius,

$$\text{number of } N_2O \text{ moles in 1 m}^3 \text{ of sample } = \frac{(101325\ )(10^{-6}\text{X})\ \text{mol}}{(8.3144)(298.15)}$$

$$\text{mass of } N_2O \text{ in 1 m}^3 \text{ of sample } = \frac{(101325\ )(10^{-6}\text{X})\ \text{mol}}{(8.3144)(298.15)} \times 44\ \text{gmol}^{-}$$

APPENDIX 4: Protocol of microbial DNA extraction

Microbial DNA extraction by using Power soil DNA isolation kit

1. 0.25 g of each soil sample was weighed for each power bead tube.
2. The sample was gently vortex for 5 seconds.
3. 60 µl of C1 solution was added.
4. Tom micro smash mixer was used to vortex for 10 minutes at 3500 rpm
5.  Centrifuged for 30 seconds at 10000 g room temperature
6. 400-500 µl of supernatant was transferred to an Eppendorf tube (1)
7. 250 µl of C2 solution was added to 2 ml tube (1) and mixed it well with supernatant using mixer and was kept cooled in the refrigerator.
8.  Centrifuged for 1 minute at 10000 g at room temperature
9. 600 µl of supernatant was taken to 2 ml tube (2) not taking the precipitation
10. 200 µl of C3 solution was added to 2 ml tube (2) and mixed it a bit, kept it cool in refrigerator.
11.  Centrifuged for 1 minute at 10000 g at room temperature
12. Avoiding precipitation, the supernatant at most 750 µl was taken to 2 ml tube (3)
13. 1200 µl of C4 solution was added and set in shaker for 5 seconds.
14. 675 µl of solution in 2 ml tube (3) was taken into spin filter tube
15.  Centrifuged for 1 minute at 10000 g at room temperature
16. Filter part was separated from the tube, and only the solution in the tube was discarded and was repeated this for 3 times (A total of 3 loads for each sample processed is required.)
17. 500 µl of C5 solution was added to spin filter
18.  Centrifuged for 30 seconds at 10000 g
19. Filter part was separated from the tube and was assembled with 2 ml tube (4)
20. 100 µl of C6 solution was added to 2 ml tube (4)
21.  Centrifuged it for 30 sec at 10000 g
22. The remaining gel in the tube was the extracted DNA.

APPENDIX 5: Protocol of gel extraction

Gel extractions by using QIAquick® Gel Extraction Kit

Before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top micro centrifuge.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.
6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
7. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

8. Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt ended ligation), let the column stand 2–5 min after addition of Buffer PE.
9. Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
10. Place QIAquick column into a clean 1.5 ml micro centrifuge tube.
11. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.
12. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

APPENDIX 6: Protocol of Plasmid DNA extraction

Protocol of Plasmid DNA extraction using the QIAprep Spin Miniprep Kit

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB medium.

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥ 5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.
4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
5. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.
6. Centrifuge for 30–60 s. Discard the flow-through.

7. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.
8. Wash QIAprep 2.0 spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
9. Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer. Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
10. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.

APPENDIX 7: One-way ANOVA output for N_2O flux data

ANOVA

redox

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36932.886	2	18466.443	64.717	0.000
Within Groups	173773.618	609	285.343		
Total	210706.503	611			

Post Hoc Tests

Multiple Comparisons

Dependent Variable:
Tukey HSD

(I) MFC		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
MFC	MFCwithextV	2.93627	1.67257	0.186	-0.9933	6.8659
	NonMFC	17.75000*	1.67257	0.000	13.8204	21.6796
MFCwithextV	MFC	-2.93627	1.67257	0.186	-6.8659	0.9933
	NonMFC	14.81373*	1.67257	0.000	10.8841	18.7433
NonMFC	MFC	-17.75000*	1.67257	0.000	-21.6796	-13.8204
	MFCwithextV	-14.81373*	1.67257	0.000	-18.7433	-10.8841

*. The mean difference is significant at the 0.05 level.

APPENDIX 8: One-way ANOVA output for soil redox data

ANOVA

redox

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36932.886	2	18466.443	64.717	0.000
Within Groups	173773.618	609	285.343		
Total	210706.503	611			

Post Hoc Tests

Multiple Comparisons

Dependent Variable:
Tukey HSD

(I) MFC		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
MFC	MFCwithextV	2.93627	1.67257	0.186	-0.9933	6.8659
	NonMFC	17.75000*	1.67257	0.000	13.8204	21.6796
MFCwithextV	MFC	-2.93627	1.67257	0.186	-6.8659	0.9933
	NonMFC	14.81373*	1.67257	0.000	10.8841	18.7433
NonMFC	MFC	-17.75000*	1.67257	0.000	-21.6796	-13.8204
	MFCwithextV	-14.81373*	1.67257	0.000	-18.7433	-10.8841

*. The mean difference is significant at the 0.05 level.