Studies on physiological characteristics of *Pseudomonas* denitrifiers isolated from post-harvest soil of dent corn Andisol farmland, and regulative approach of those nitrous oxide (N_2O) emitters relevant to active N_2O efflux from the soil

(収穫後デントコーン畑地黒ボク土壌から分離した *Pseudomonas* 属脱窒細菌群の生理学的特徴と，その土壌の高い亜酸化窒素（N_2O）放出を担う N_2O 生成細菌群の制御に関する研究)

Ph.D. Dissertation
(The *Special* Postgraduate *Program* in Biosphere Sustainability Science)

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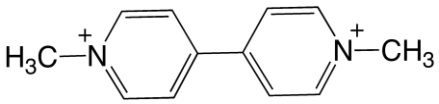
Abbreviations

BLAST	basic local alignment search tool
CFU	colony forming unit
d	Day
DDBJ	DNA databank of Japan
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	dimethyl sulfoxide
DNRA	Dissimilatory nitrate reduction to ammonium
e.g.	for example (*exempli gratia*)
et al.	and others (*et alii*)
g	Gram
GC	gas chromatography
mM	Millimolar concentration
min	Minute
ml	Milliliter
NCBI	national center for biotechnology information
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rpm	rotations per minute
TLC	thin layer chromatography
µl	Microliter
µM	Micromolar concentration

Structures of compounds used



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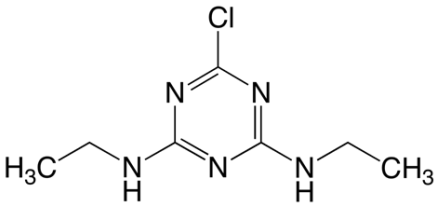

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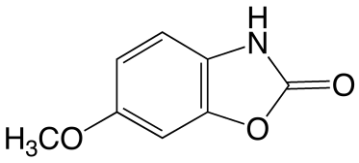

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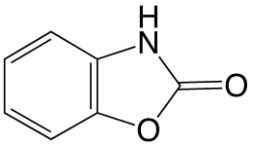

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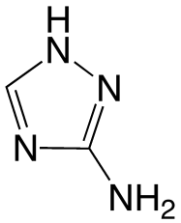
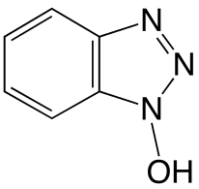


amitrole



1-hydroxy-1*H*-benzotriazole (HOBt)

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Summary

Nitrous oxide (N_2O) is an active greenhouse gas that causes more than 7% of global warming and contributes to the depletion of the ozone layer. Emphatically, denitrification is the main biological process responsible for nitrous oxide emission, a microbial respiratory process under limited oxygen. Denitrification by microorganisms plays an important role in nitrogen circulation. Both the denitrification rates and N_2O emission produced by denitrifying microorganisms can vary depending on numerous environmental factors, such as pH, carbon, NO_3^- and NO_2^- availability, soil moisture, soil pore structure, aeration, temperature, freezing-thawing events, and drying-wetting events. Owing to these factors, it is important to find an effective way to regulate N_2O emission in soil. Andisol from the Hokkaido University Shizunai Experimental Livestock Farm in Shinhidaka, Hokkaido, Japan, is considered to be relatively active efflux area of N_2O emission.

To develop novel approaches to control N_2O emission, N_2O-emitting bacteria were screened from culturable soil bacteria isolated from Andisol from a corn farm at the Experimental Livestock Farm using a reproducible N_2O emission assay culturing in a soilless, gellan gum-base soft gel medium. In our study, the characteristics of N_2O emitters are described and the related biological methods to regulate N_2O emission are also summarized.

1. **Isolation of N_2O-emittable *Pseudomonas* spp. and their characteristics**

Using dent corn farm soils collected at the post-harvest time (autumn 2011) and the pre-tillage time in spring (April 2012) as the inoculants, supernatant of the soil suspensions was cultured in an N_2O assay medium containing an excessive amount of KNO_3 (3.6 mg mL^{-1} medium), and those showing active N_2O emission were further screened on modified Winogradsky's agar plate for their isolation. Consequently, 4 strains from the gel cultures of post harvest farm soils and 6 strains from the cultures of pre-tillage soils were selectively obtained from the 76 colonies isolated. All of these active N_2O emitters were identified as *Pseudomonas* sp. by means of sequence

determination followed by homology search on their 16S rRNA gene region. An acetylene inhibition test and a PCR assay for detection of *nosZ* gene showed that 6 strains (3 from the post-harvest soil and 3 from the pre-tillage soil) among the 10 isolates of N_2O emitters were likely incomplete denitrifiers, of which *nosZ* gene does not function. N_2O emitters showing incomplete denitrifier-like behaviors, particularly those of post-harvest soil showed high responses to 0.05-0.5% sucrose to produce more amount of N_2O. Accordingly, it was likely that highly saprophytic pseudomonad denitrifiers mainly contributed to remarkably high N_2O emission of the corn farm soil in summer season with sufficient carbon sources. Conversely, active N_2O emitters in the soil before tillage were influenced by water soluble organic carbons in soil provided by corn roots due to active but incomplete nitrate respiration in soil along with NO_3^- and NH_4^+ applied as chemical fertilizer and manure input.

2. Effects of C- and N-sources on microbial community structures and N_2O emission

Andisol soil (5 g) obtained from a post-harvest dent corn farm at the Hokkaido University Shizunai Livestock Farm emitted N_2O at rate of 6 ng d^{-1}. In contrast, the same soil supplemented with 1.5 mM sucrose and 0.1 mM inorganic nitrogen sources (regardless of NO_3^-, NH_4^+, or both) produced 20- to 30-fold higher N_2O than the control. Denaturing gradient gel electrophoresis (DGGE), however, no significant differences in bacterial communities were observed among the treated soils and the control, irrespective of the presence or absence of C- and/or N-source supplementation. It was, hence, suggested that pseudomonad denitrifiers were selected effectively by the trapping culture supplemented with the excessive amount of KNO_3. Consequently, supernatants (100 μl) of the soil suspension (1 mg ml^{-1}) or soil itself (10 mg per vial) were cultured in soft gel medium supplemented with 0.05-0.5% sucrose as a carbon source with combined with 5 mM NO_3^-, NH_4^+, NH_4NO_3, or any additional nitrogen sources and N_2O produced were measured. Developed bacterial community structures in the soft gel culture were then monitored by 16S rRNA gene-targeted PCR-DGGE analysis. The pseudomonad denitrifiers became dominant in the culture medium

supplemented with the excessive KNO_3, while *Burkholderia* and *Arthrobacter* emerged in the culture upon enrichment of sucrose and $(NH_4)_2SO_4$, respectively. Addition of sucrose accelerated N_2O emission from the culture, while NH_4^+ led to suppression of the N_2O emission.

3. Effects of herbicides and their related chemicals on N_2O emitters

Some commercial herbicides, a representative antimicrobial secondary metabolite of corn, 6-methoxy-2-benzoxazolone (MBOA), and *N*-heterocyclic compounds structurally related to MBOA were examined their effects on N_2O-emitting soil bacteria. In the N_2O emission assay, two N_2O-emitting eubacteria, two incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B and 10CFM5-2D (both isolated from post-harvest Andisol corn farmland in Hokkaido), were used. It was found that methyl viologen dichloride (Paraquat®) at 2 μM significantly repressed N_2O emission by the active denitrifying bacteria. MBOA also repressed pseudomonad denitrifiers at 10 μM. Other herbicides such as simazine (6-chloro-*N*,*N'*-diethyl-1,3,5-triazine-2,4-diamine) and amitrole (3-amino-1,2,4-triazole) accelerated N_2O emission by *Pseudomonas* sp. 10CFM5-1B at 2 or 10 μM. This study suggested that methyl viologen dichloride may have somehow contributed to the repression of global warming by suppressing N_2O production from farm soils in a global scale. Some herbicides, including amitrole and other triazole-type chemicals, may instead have potentials to activate N_2O emission from the fertilized soils.

Chapter 1

Introduction

In this chapter, the background of nitrogen cycle, especially nitrification and denitrification processes related with the emission of N_2O, an active greenhouse gas that leads to the depletion of the ozone layer is described. Emphatically, denitrification, a microbial respiratory process under limited oxygen, is the main biological process responsible for N_2O emission. As the natural variations, agroecosystems are characterized by means of numerous practices, such as fertilization and pesticide application, which can influence denitrification progression and N_2O emission. In order to reduce the N_2O production due to denitrification in soil, it is important to understand the factors which can activate the N_2O emission by soil N_2O emitters. The physiological characteristics of active N_2O emitters such as, *Leptotheix* sp., *Paenibacillus* sp., *and Streptomyces* sp. isolated from Andisol farm soils were also illustrated.

Finally, the aim of this research is to regulate the N_2O emission by N_2O emitters in agricultural soil, and the approaches and objectives of each theme combined with the implication and significance, are also stated in this section.

1.1 The biological nitrogen cycle in global scale

1.1.1 The roles of nitrogen in living organisms

The global nitrogen cycle is one of the most important nutrient cycles in terrestrial and marine ecosystems. The roles of nitrogen in living organisms can be grouped into two general categories: assimilation, i.e., the acquisition of mineral nitrogen for the catabolism and incorporation into biomass, and dissimilation, which designates processes that are associated with metabolisms, including respiration for acquisition of energy (Thamdrup, 2012). Nitrogen often comes from fertilizer

application, and only legumes such as soybean and alfalfa can convert atmospheric N_2 to plant-available forms via a symbiotic biological process of the plant roots in association with nodulation bacteria including *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium* (Mokhele et al., 2012). Nitrogen assimilation into carbon skeletons represents the important physiological process of the plant growth and development. Plant-available inorganic forms of N, include nitrate, nitrite, and ammonium, is assimilated into amino acids, namely glutamate, glutamine, and asparagines, all of which play an important role as N transportable compounds in plants (Lea and Miflin, 2003). In the rhizosphere, the root can release oxygen and exudates that greatly influence local redox potential, the density of microbial population and their biological activities which in turn can interconvert soil N forms, including those derived from chemical and organic fertilizer (Xu et al., 2012).

On global scale, the biological nitrogen cycle of mineral nitrogen converted into N_2 through dissimilatory transformation known as denitrification process and their regulations are important in both natural and anthropogenic agricultural ecosystems.

1.1.2 Microbiological processes of nitrogen cycle

Nitrogen cycle involves four microbiological processes: nitrogen fixation, nitrogen mineralization and immobilization, nitrification, and denitrification (Hayatsu et al., 2008). Biological nitrogen fixation, representing major source of N supply in natural vegetation soils or even in agricultural soils, is essential for all forms of life because nitrogen is required to biosynthesize basic piece of block in plants, animals and other lives.

Mineralization results in an increase of plant-available forms of N in the soil, while immobilization results in their decrease. Through the mineralization of nitrogen, microbes break down organic nitrogen containing compounds and release N as ammonium (NH_4^+). Plants from a variety of habitats can uptake amino acids and other organic N forms; mycorrhizas play a role in this uptake by absorbing amino acids, amino sugars, peptides, proteins, and chitin as N sources. It is also known that

mineralization and immobilization are occurring at the same time. One group of microbes might consume a protein-rich and nitrogen-rich piece of organic matters (the process of N-mineralization), while another group might consume detritus that is rich in C but low in N (the process of immobilizing N). Because all heterotrophic soil organisms consume organic materials for energy and carbon sources, while immobilize and/or mineralize N as a by-product, nitrogen mineralization and immobilization abilities are widely distributed throughout heterotrophic microorganisms (Robertson and Groffman, 2007).

Nitrification is an important part of the nitrogen cycle, because nitrate is the preferred chemical form of nitrogen uptake for a large number of plants. Nitrification is an aerobic microbial process that oxidizes ammonium (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-) by specialized bacteria. Because nitrate and nitrite are much more mobile in soils than ammonium, nitrification can be recognized as a main process to mobilize nitrogen. Most available nitrate and nitrite are uptaken by plants, but these mineral forms of nitrogen potentially leach out from the ecosystem.

Denitrification, the reverse process of nitrification, is the respiratory anarobic reduction of NO_3^- via NO_2^-, NO, and nitrous oxide (N_2O) to dinitrogen (N_2). NO, N_2O and N_2 are gaseous compounds and generally they are not readily available for microbial growth; therefore they are typically released to the atmosphere. Denitrogen gas makes up over 70% of atmospheric gases, thus the release of N_2 to the atmosphere is begin. Under high organic matter and anaerobic soil conditions in the soils, the denitrification rates are increased. Denitrification leads to loss of nitrogen from the soil which results in the depletion of an essential nutrient for plant growth.

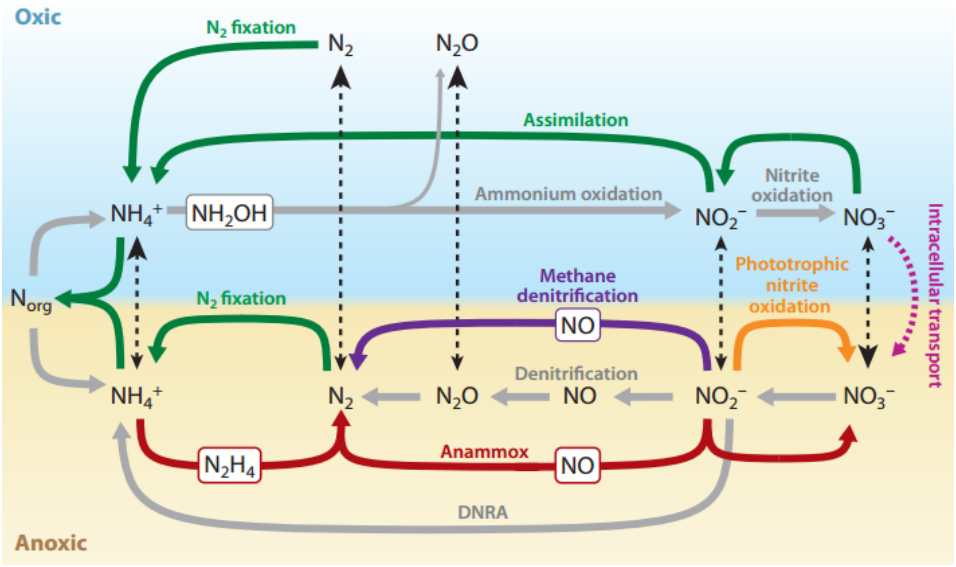
The process of denitrification can occur by two pathways. The dissimilative nitrate reduction pathway requires anoxic conditions and results in the liberation of nitrogen gas from the water column (patraReed et al., 1988). Under aerobic conditions, denitrification resulted in the assimilative pathway or accumulation of nitrogen into biomass (Bitton, 1994). It is desirable to encourage the dissimilative pathway of denitrification, so that nitrogen may be completely removed from the system as

gaseous form N_2 rather than simply recycled through the system in biomass. In order to occur for this event, insufficient gaseous or dissolved oxygen must be present so that the bacteria use the nitrate rather than the oxygen as an electron acceptor. The rate of the denitrification reaction is relatively fast when there is no free oxygen presented (< 0.5 mg/L). The denitrification rate drops to zero when the dissolved oxygen level reaches 2.0 mg/L. Although denitrification is an undesirable reaction from agricultural productivity, it is of major ecological importance.

Nitrate dissimilation processes contain two pathways, denitrification and dissimilatory nitrate reduction to ammonium (DNRA), the latter of which is also termed fermentative NO_3^- reduction, NO_3^- ammonification, or fermentative ammonification. The occurrence and importance of DNRA is generally not considered because there is general agreement that the denitrification process takes place in many soils (Rütting et al., 2011). Two approaches have been used to investigate DNRA in soil, (1) microbiological techniques to identify soil microorganisms capable of DNRA and (2) ^{15}N tracing to elucidate the occurrence of DNRA and to quantify gross DNRA rates. As the down of nitrogen metabolic study, Woods showed that DNRA occurs in common soil bacteria like *Clostridium welchii* and got the conclusion that DNRA must be seriously considered in assessing the importance of the oxidation of NH_3 to NO_3 (Woods, 1938). This view was evidenced later using ^{15}N tracing techniques, leading to argument about the serious challenge to the prevalent view that denitrification accounts for essentially all NO_3^- dissimilation in anaerobic soils (Stanford et al., 1975).

In recent years, studies on N cycle have increasingly targeted on DNRA in various ecosystems. Various environmental factors influence DNRA in soil, such as redox state of soils (Matheson et al., 2002), organic carbon source (Mohan et al., 2004; Morley and Baggs, 2010), pH (Woods, 1938; Davidsson and Stahl, 2000; Šimek and Cooper, 2002), and so on. Taken together, the oxidation status and C/NO_3^- ratio were reported to be the most important factors regulating the DNRA in soil, while the effect of pH was not consistent. The presence of roots alters the activity and abundance of dissimilatory NO_3^- reducers in soils, as a consequence of altered substrate and oxygen

availability (Philippot et al., 2009). However, so far no study has investigated the direct effect of plants on DNRA in upland soil, only some information is available for wetland/freshwater plants.



(Adapted from Thamdrup, 2012)

Figure 1.1 Major transformations in the nitrogen cycle.

The process of assimilation is shown as green thick arrows, whereas dissimilation process is shown by gray thick arrows. Atmospheric dinitrogen (N_2) can be deposited in the soil followed by fixation by soil nitrogen-fixers and is subsequently converted to NH_4^+. Alternatively, reactive forms of nitrogen can be deposited in precipitation or as dry deposition. Sources of N_2O, including fixed N_2, can also be released from organic residues of dead plants and animals. DNRA, dissimilatory nitrate reduction to ammonium.

1.1.3 Microorganisms play important roles in the cycling of nitrogen

Nitrogen-fixing bacteria

Atmospheric nitrogen remove as dinitrogen (N_2) can be fixed by N_2-fixing bacteria and archaea into inorganic nitrogen compounds, such as ammonium (NH_4^+), which is incorporated into amino acids and utilized by plants, to be incorporated into biomass. Other prokaryotes as well as all the eukaryotes required fixed nitrogen or organic nitrogen for assimilation (Thamdrup, 2012). All organisms that can reduce

dinitrogen to ammonia need an enzyme complex, nitrogenase. The nitrogenases are irreversibly inactivated by oxygen, while the process of nitrogen fixation requires a large amount of ATP (Postgate, 1984; Zahran, 1999). A wide range of prokaryotic microorganisms have the ability to fix nitrogen: about 87 species in 2 genera of archaea, 38 genera of eubacteria, and 20 genera of cyanobacteria, have been identified as diazotrophic microorganisms that can fix nitrogen (Postgate, 1984; Zahran et al., 1995).

Nitrifying bacteria and archaea

Nitrificaion, the oxidation of reduced forms of nitrogen to nitrate, carried out by three microbial groups: autotrophic ammonia oxidizers, autotrophic nitrite oxidizers, and heterotrophic nitrifiers. Nitrifying bacteria which can use some of the electrons from oxidation of ammonium and nitrite to reduce CO_2 and build biomass are autotrophic. Many microorganisms and plants require ammonium for their growth, while others assimilate nitrate. For both groups, nitrification is important in regulating the supply or loss of nitrogen from the environment. Autotrophic ammonia oxidizers (AOB) are gram-negative bacteria, traditionally placed within the Nitrobacteriaceae and characterized by their ability to oxidize ammonia. Nitrite oxidizers (NOB) are classified into four genera within the proteobacteria: *Nitrobacter, Nitrococcus, Nitrospira,* and *Nitrospina*. Heterotrophic nitrification is the oxidation of NH_3^+ and organic nitrogen as reduced forms, to nitrate, by a wide range of fungi and heterotrophic bateria. In some microorganisms, the mechanism of N-oxidation is similar to that in autotrophic ammnia oxidizers, and in some strains of fungi, the heterotrophic nitrifiers is linked to aerobic denitrification.

Denitrifying bacteria

Most of the denitrifiers that consume organic substrates are heterotrophic. Denitrying microbial populations have been extensively profiled in soils, waster treatment system, and marine environments (Liu et al., 2003; Heylen et al., 2006; Philippot et al., 2009). Most eubacteria with denitrification trait belong to unrelated

systematic affiliations, in addition, some archaea and even the mitochondria of some fungi also exhibit the ability of denitrification (Philippot, 2002; Hayatsu et al., 2008; Fang et al., 2010). *Pseudomonas* species are generally presumed to be the dominant microorganisms through the denitrificaiton process (Lazarova et al., 1992) whereas various species such as *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium and Hyphomicrobium* are responsible for denitrification in soil (Lim et al., 2005). Even though the diversity of denitrifiers is very high, it is likely that several unknown denitrifying microorganisms are still present in nature and contribute to the denitrification in N cycle. For instance, a benthic foraminifer *Globobulimina pseudospinescens* which uniquely accumulates NO_3^- in intracellular stores, can perform nitrate respiration to yield N_2 gas (Risgaard-Petersen et al., 2006).

Soil microorganisms involved in DNRA

The capabilities for NO_3^- respiration and DNRA are widely spread throughtout bacteria Kingdom (Simon, 2002; Philippot, 2005). Several genera of soil DNRA bacteria have been reported, such as obligate anaerobes (*Clostridium*), facultative anaerobes (*Citrobacter, Enterobacter, Erwini, Escherichia, Klebsiella*) and aerobes (*Bacillus, Pseudomonas*) (Tanner, 1989). In addition, a strain of *Arthrobacter*, a worldwide genus in soil which is regarded as an obligate aerobe, exhibited DNRA under anaerobic incubation (Eschbach et al., 2003). The capability for DNRA is also widely distributed throughout common soil fungi, mostly belonging to the ascomycota; and denitrification and DNRA are alternatively expressed in a common soil fungus (*Fusarium oxysporum*) depending on oxygen status and available C source (Zhou et al., 2002). None of the bacteria have been well established to process capabilities of both denitrfication and DNRA, before a study provided evidence by growth tests that two new *Paenibacillus* species, including one isolated from fen soil isolate, showed a versatile metabolism and were capable of heterotrophic nitrification, DNRA and denitrification (Behrendt et al., 2010). Conversely, many microorganisms conducing DNRA produce N_2O, which is involved in a detoxification mechanism in

order to avoid excessive concentrations of NO_2^- (Kaspar, 1982). All the results discussed are based on culturable microorganisms and the activity of a DNRA bacterium was shown to differ between pure culture (a DNRA isolate, *Enterobacter amnigenus*) and soil inoculation (nonsterilised soil samples inoculated with *Enterobacter amnigenus*) (Fazzolari et al., 1990). Investigation of the bacteria using functional genes as an index of microbial diversity and enzyme activities could provide a potent tool for investigating and comparing the DNRA and denitrification activity and potentials of the soil microbial communities in soils.

1.2 Nitrous oxide production in nitrogen cycle associated with agriculture

1.2.1 Main processes of N_2O production

Nitrous oxide (N_2O) is an active greenhouse gas that causes more than 7% of global warming (IPCC 2007) and contributes to the depletion of the ozone layer (Ravishankara et al., 2009). Global anthropogenic sources of N_2O include agriculture and industy, which generate the gas through biomass burning, indirect emissions from reactive nitrogen leaching, runoff, and atmospheric deposition (IPCC 2001). Particullarly, agricultural soil plays a dominant role in N_2O emission because of the widespread use of nitrogenous fertilizers and manure (Galloway et al., 2004; Reay et al., 2012). A recent report showed that 70% of the annual anthropogenic N_2O yield is produced in agricultural farm soil by the processes of nitrification and denitrification (Rojas-Oropeza et al., 2012).

N_2O production from agricultural soil is regulated by four main microbial processes: nitrification (ammonium oxidation) (Bremner, 1997), nitrifier denitrification (nitrite reduction) (Wrage et al., 2001), denitrification (Groffman et al., 2006) and dissimilatory nitrate reduction to ammonium (nitrate reduction). These processes have been reported to occur simultaneously at different micro-sites in soil (Templer et al., 2008). Generally, it is assumed that biological denitrification is the

most important process in global N circulation and N_2O production, and it has been shown that almost 90% of N_2O emitted from soil results from denitrification rather than nitrification in nitrogen cycle in farm soils (Bateman and Baggs, 2005).

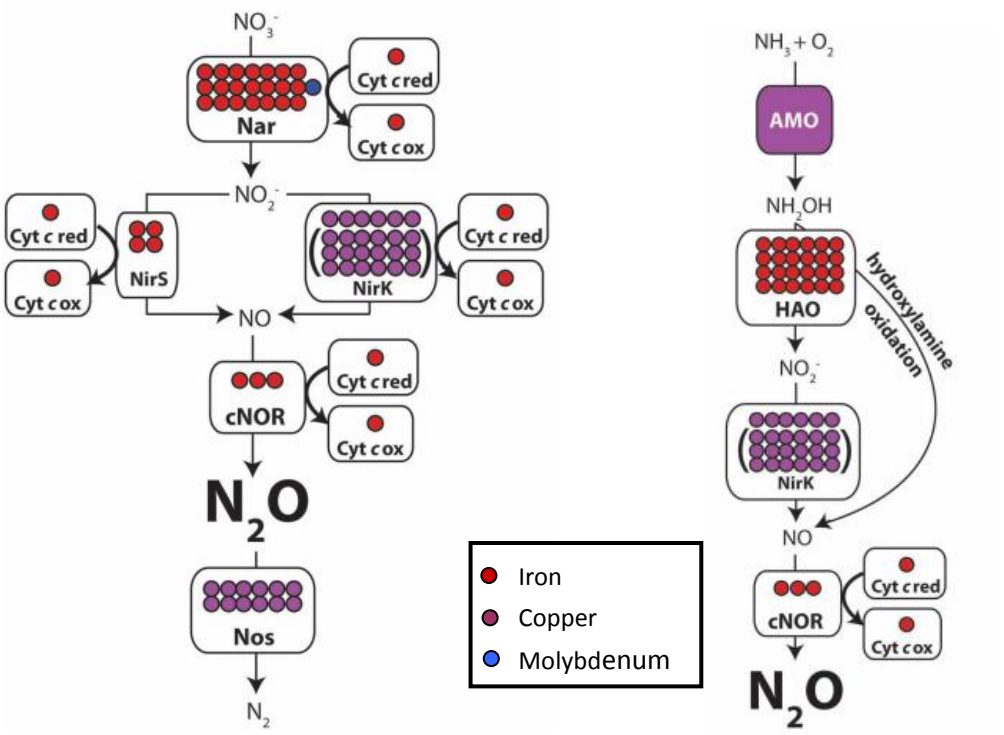
Microorganisms capable of DNRA can also produce N_2O, which is a detoxification mechanism, in order to avoid high concentrations of NO_3^- (Kaspar, 1982) (Fig. 1). In batch cultures with defined media, NO_2^- reduction to NH_4^+ by soil *Citrobacter* sp. was favored in presence of high glucose and low NO_3^- concentrations, while N_2O production was greatest at high glucose and intermediate NO_3^- concentrations (Smith, 1982). Several microorganisms, such as *Bacillus*, *Citrobacter*, were able to simultaneously produce NH_4^+ and N_2O via dissimilatory pathways, and production of N_2O was not restricted to aerobic or anaerobic conditions (Bleakley and Tiedje, 1982). Similarly, in an anaerobic batch incubation study, all the DNRA isolates form three different soils exhibited N_2O production, which accounted for 5%-10% of added NO_3^- (Smith and Zimmerman, 1981). There was evidence that DNRA was occurring at higher pH values, possibly as a mechanism to reduce harmful NO_2^-, became a more important process for N_2O production (Stevens et al., 1998). Recently study shows that *Bacillus vireti* has a versatile metabolism, as predicted from its genotype, indeed it can synthesize a functional NO_3^- reductase, NH_4^+ forming NO_2^- reductase, NO reductase and N_2O reductase (to form NO_2^-, NH_4^+, N_2O and N_2, respectively) (Mania et al., 2014).

1.2.2 Functional genes involved in denitrification

Biological denitrification, the primary N_2O producing process in the nitrogen cycle (Bateman and Baggs, 2005), requires four enzymes for reduction of NO_3^- to N_2, Each enzyme uses active metal as a redox active metal cofactor show them in Fig. 1.2. Two types of molybdoenzymes: a membrane bound (Nar) and a periplasmic (Nap) NO_3^- reductase catalyze the first step of the pathway, the reduction of NO_3^- to NO_2^- (Roussel-Delif et al., 2005). The reduction of soluble NO_2^- into gaseous nitric oxide (NO) can be catalyzed by evolutionary unrelated enzymes that are different in terms

of structure and of prosthetic metals-a cytochrome *cd1* (NirS) and a copper nitrite reductase (NirK) (Glockner et al., 1993). The nitrite reductase genes (*nirS* and *nirK*) are functional marker genes of denitrifying bacteria, since this physiological group is widespread among phylogenetically unrelated groups. The reduction of NO into N_2O is also catalyzed by two types of enzymes: one NO reductase receives the electrons from cytochrome *c* or pseudoazurin (cNor) while the other from a quinol pol (qNor). The last step of denitrification, reduction of N_2O into dinitrogen gas (N_2), is performed by a multi-copper homodimeric N_2O reductase (*nosZ*), which locates in the periplasm of Gram-negative bacteria (Wood et al., 2001; Tavares et al., 2006). However, not all the denitrifiers process *nosZ* gene which is required for complete the denitrification process (Zumft, 1997). Denitrification process can act as both a source and a sink of N_2O, depending on whether N_2O is produced as the intermediate or the end product (Chapuis-Lardy et al., 2007).

Classic denitrification **Nitrifier denitrification and hydroxylamine oxidation**



(Adapted from Glass, 2012)

Figure1. 2 The process of denitrification.

Denitrification process was catalyzed by the nitrate (NO_3^-), nitrite (NO_2^-), and nitrous oxide (N_2O) reductases, and the name of the genes encoding the corresponding catalytic subunits. Each circle represents one metal atom. Paraentheses show varing metal content of a given enzyme. Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; Nar, dissimilatory nitrate reductase; NirS, Fe-nitrite reductase; NirS, Cu-nitrite reductase; cNOR, nitric oxide reductase; Nos, nitrous oxide reductase.

1.2.3 Studies on N_2O-emitting bacteria in soil

Major N_2O emitting bacteria from tropical peat soils in Central Kalimantan, Indonesia, one of the most active N_2O emitting sites in the world (Takakai et al., 2006), were successfully isolated. Using a soilless culture system mimicking tropical acidic peat soil, which contained 3 mg of gellan gum and 0.5 mg NO_3^--KNO_3 per

gram of medium, an acid-tolerant *Janthinobacterium* sp. strain A1-13 isolated from soil of an arable land was characterized as one of the most active N_2O-emitting bacteria in this region. Besides, *Burkhodleria tropica* and *Burkhodleria cepacia* were also produced significant amounts of N_2O (Hashidoko et al., 2008). With the increasing amount of carbon source glucose to the standard medium, *Janthinobacterium* sp. strain A1-13 showed remarkable increase of N_2O emission. The N_2O production was greatest at pH 3.8 in acidic tolerant *Janthinobacterium* sp. strain A1-13, while N_2O emission is generally suppressed in acidic soil (Daum and Schenk, 1998), suggested that moderately to strongly acidic soils having a high buffering capacity to increase N_2O emission.

Furthermore, the active N_2O emitters *Leptotheix* sp., *Paenibacillus* sp., *and Streptomyces* sp. isolated from an Andisol in Shizunai Experimental Livestock Farm in Hokkaido and N_2O emitting performance of these bacteria are distinguishable from those isolated from the tropical peat soil in both quality and quantity (Takeda et al., 2012). The N_2O emitting *Janthinobacterium* sp. strain A1-13 exhibited 50-500 fold higher activity than N_2O emitters isolated from the Andisol farmland (Hashidoko et al., 2008). N_2O production from all the N_2O emitters isolated from Andisol farmland was much more active within a weakly acidic region (pH 4.5-5.0) than neutral regions (pH 5.5-7.0). However, unlike N_2O emitters isolated from tropical peat soils, pure-cultured bacteria did not show any significant responses to a high concentration of NO_3^- even in the presence of appropriate concentrations of sugar as the carbon source. In the medium for the sugar-supplemented assay, *Leptothrix* sp. P3-15D exhibited approximately 6-fold enhancement of N_2O emission in the medium supplemented with 0.5% sucrose, of which acceleration effect was much smaller than that observed for *Janthinobacterium* sp. A1-13 (60-100 fold).

1.2.4 Stimulation of denitrification by root exudates

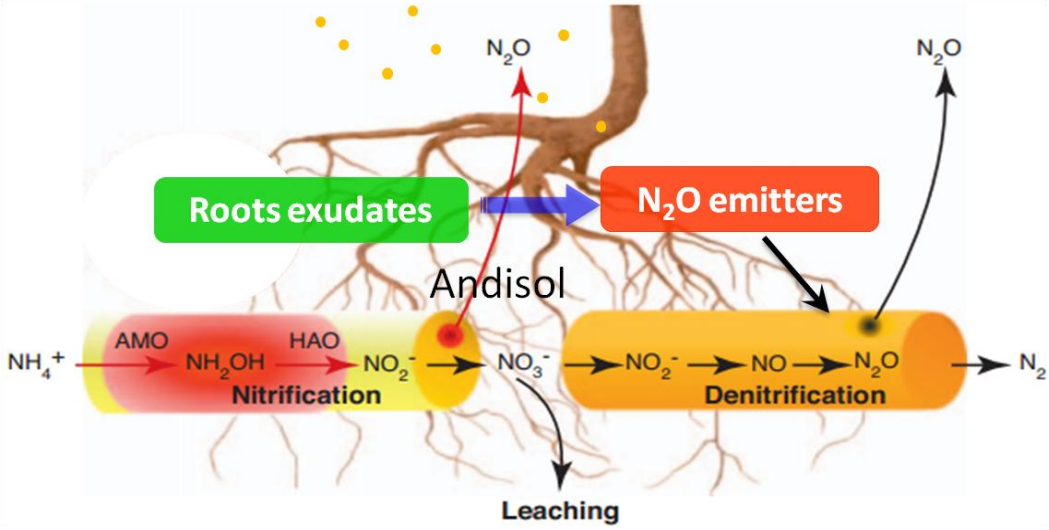
The activity of denitrifying communities is influenced by plant species, owing to the differences in quality and quantity of chemical compounds in the root exudates

(Burgmann et al., 2005; Henry et al., 2008).

The higher potentials of denitrification are associated with bigger root mass in a lysimeters study with various forage plants. Differences in the denitrification rates between small grains (barley, wheat, and oats) and grasses were also reported. So far, the agricultural crop, barley, has received the greatest attention. The denitrification rates with planted barley increased with 2-22 times compared with the unplanted pots (Klemedtsson et al., 1987). Vinter *et al.* (Vinther, 1984) demonstrated that the positive correlation of increased denitrification rates in barley rhizosphere with soil NO_3^- concentration. The similar results were observed by Mahmood et al (Mahmood et al., 1997), who carried out a field experiments to examine the effect of maize plants on denitrification. The presence of maize plants results in 2.5 times increase in denitrification at higher NO_3^- (7-19 μg N g^{-1} dry soil) levels, whereas at low soil NO_3^- levels (1-4 μg N g^{-1} dry soil) the denitrification showed nearly 1.4 times increase. The higher denitrification rates were observed in the unplanted soil, compared with planted soil at late maize growth stage with a limiting amount of NO_3^- (Qian et al., 1997). It was concluded that these neutral or negative effects of plant roots on denitrification were attributed to NO_3^- depletion around roots.

The major factors regulating denitrification: nitrate concentration (via plant N-assimilation), oxygen partial pressure (via soil moisture and soil pore size) and carbon availability (via exudation from the root surface) (Woldendorp, 1962; Moulton and Montie, 1979) can be modified in the rhizosphere of plants. Carbon, an important factor to regulate denitrification, is probably responsible for the stimulation effect of plants on denitrification activity of the rhizospheric microorganisms. Several studies have focus on the effect of different organic substrates on denitrification. The organic compounds released by living roots can directly affect denitrification by providing an additional source of electron donor. It thus seems that root exudates are likely to influence the microbial process denitrification. Early in 1962, Woldendorp had showed that the living roots stimulated denitrification (Woldendorp, 1962; Klemedtsson et al., 1987). The carbon derived from plants roots is highly variable among mucilage, exudates, root cap cells and so on. Root exudates are low molecular

weight compounds such as sugars, amino acids, and organic acids, whereas the mucilage is composed of high molecular weight polysaccharides, consisted of arabinose, galactose, fucose, glucose, and xylose. Addition of 70 µg C g^{-1} dry soil of maize mucilage into agriculture soil increased 2.8 times of denitrification compared with water addition (Mounier et al., 2004). Similarly, daily addition of different mixtures of artificial root exudates mimicking maize root exudates greatly stimulated denitrification rates (Henry et al., 2008) .



(Modified figure from Philippot, 2011)

Figure 1.3 Effect of roots exudates on nitrification and denitrification

Plant can both stimulate and inhibit the process of nitrification. The stimulation is probably due to increased organic matter that in turn enhances N turnover. The brachiolactone present in the root exudates of *Bracharria humidicola* blocks both the ammonia mono-oxygenase and the bydroxylamine oxidoreductase in ammonia oxidizers. Plant can also regulate denitrification, i.e. NO_3^- concentration (via plant N-assimilation), O_2 partial pressure (via root respiration) and carbon availability (via rhizodeposition).

Denitrification activity and denitrifying bacterial communities showed a significant difference among below grass tuffs of three major plant species. Further studies showed that organic carbons released from roots affect the diversity of microbial communities such as specific community diazotrophs (Burgmann et al., 2005) and Pseudomonas (Lugtenberg et al., 1999). Qian et al argued that root-derived C influences soil microbial activities that regulate nitrogen transformation via denitrification in soil (Qian et al., 1997).

1.3 Factors affecting denitrification rates and N_2O emission

1.3.1 Impact of pesticides on soil microorganisms

In present-day soil, increasing use of pesticides has become a cause of concern due to their effect on the composition and function of soil microoganisms (De Andrea et al., 2003; Baxter and Cummings, 2008). This effect is controlled by numerous environmental factors in addition to the persistence, concentration, and toxicity of the applied pesticide and its bioavailability (Abdelmallek et al., 1994).

Other studies have also shown that application of certain pesticides influences microbial and enzymatic reactions, including mineralization of organic matter, nitrification, denitrification, and ammonification (Mori et al., 2008; Green et al., 2010). Application of mefenoxam and mefenoxan+copper increased nitrification activity and indicated higher copy numbers of *amoA* (a functional molecular marker for β-subgroup ammonia-oxidizing bacteria) gene in the latter after 60 days of the pesticide application (Demanou et al., 2006).

Only few studies investigated the effect of pesticides on the size and the structure of the denitrifier community. The effects of carbofuran, carbendazim, and butachlor on the population size of denitrifying bacteria and their activity in different Chinese paddy soils were investigated (Table. 1.1). Lower concentrations of the pesticides (1 μg g^{-1} dry weight soil) increased the population size and activity, whereas higher concentrations reduced both parameters. Increased numbers of denitrifiers were

observed after addition of 50-300 μg g^{-1} soil of malathion (Gonzalezlopez et al., 1993). Addition of herbicide topogard in soil with varying pH increased denitrifiers, and this effect was likely to be dependent on soil pH (Kara et al., 2004). Conversely, another study reported that the community structure and activity of the NO_3^--reducing bacteria in a maize field were not affacted by atrazine or glyphosate (Philippot and Hallin, 2006).

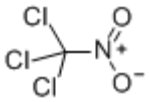
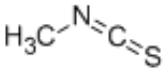
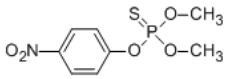
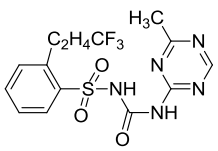
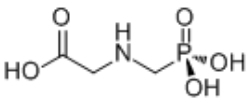
1.3.2 Effect of pesticides on N_2O emission

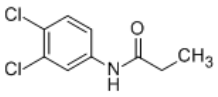
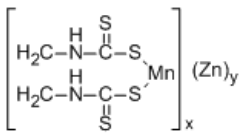
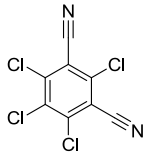
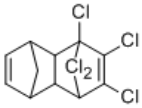
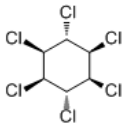
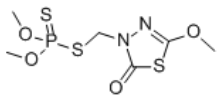
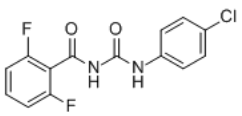
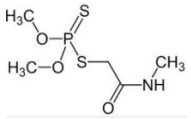
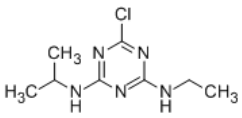
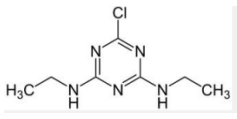
Studies on the positive and/or negative effects of pesticides on N_2O emission by denitrifying soil bacteria have been performed to determine the effects of pesticides on soil microbial biomass and soil respiration (Ingram et al., 2005; Pampulha and Oliveira, 2006; Wang et al., 2007). The potential agrochemical impact on N_2O production by soil fumigation with chloropincrin and methyl isothiocyanate (Table. 1.1) was separately examined, and it showed that both stimulated N_2O production (Spokas et al., 2005; Spokas et al., 2006). It has also been reported that methyl parathion can serve as a C-substrate and e^- donor thereby increasing NO_3^- reduction and consequently emission of N_2O (Blanco-Jarvio et al., 2011) and also reduces the diversity of the *nirK* gene affecting N_2O production (Rojas-Oropeza et al., 2012). Conversely, the herbicides prosulfuron, glyphosate, and propanil, and the fungicides mancozeb and chlorothalonil, suppress N_2O emission in soil due to the inhibition of nitrification and/or denitrification (Kinney et al., 2005; Kyaw and Toyota, 2007). The herbicide atrazine and an insectidice dimethoate completely inhibited growth and biologicl activity of *X. autotrophicus,* while the other tested pesticides (eg. Aldrin, lindane, methylparathion, methidation, simazine, captan, and diflubenzuron) delayed the growth of strain *X. autotrophicus* (Saez et al., 2006). N_2O emission was strongly inhibited by several pesticides (aldrin, lindane, methyl parathion, methidathion, and diflubenzuron), while dimethoate, atrazine, and simazine inhibited the denitrifying activity of the strain (Saez et al., 2006).

The impact of pesticides on denitrification activity in soil is likely to be

dependent on the soil type, the concentration and nature (pure active ingredient or formulated preparation) of the applied pesticide, the climate conditions, and the way for degradation. The application of pesticides can inhibite the process of denitrification probably attributing to cell death or cell inactivation. However, it can also stimulate this process attributing to (1) the pesticide used as an electron donor by denitrifiers; (2) the dead microorganisms available as carbon source of denitrification; (3) an unspecific stress response (Philippot et al., 2007).

Table 1.1 Effect of pesticides on N_2O emission

Pesticide	Structure formula	Concentration	Effect	Reference
Chloropincrin (CP)		68 μg g^{-1} (field application rates)	Increased N_2O emission	Spokas *et al.*, 2005
methyl isothiocyanate (MITC)		55 μg g^{-1} (field application rates)	Increased N_2O emission	Spokas *et al.*, 2005
methyl parathion		10g soil amended with 2.6 ml methylparathion	Increased N_2O emission	Blanco-Jarvio *et al.*, 2011
prosulfuron		2.0 ng g^{-1} - 1.0 μg g^{-1} (0.71 μg g^{-1} normal field appliction concentration)	Inhibited N_2O production	Kinney *et al.*, 2005
glyphosate		5 nl g^{-1} (field rate)	Suppressed N_2O production	Kyaw and Toyota, 2007

propanil		5 nl g^{-1} (field rate)	Suppressed N_2O production	Kyaw and Toyota, 2007
mancozeb		52 ng g^{-1} – 26 µg g^{-1} (2.6 µg g^{-1} normal field appliction concentration)	Inhibited N_2O production	Kinney *et al*., 2005
chlorothalonil		1.9 nl g^{-1} - 0.93 µl g^{-1} (93 nl g^{-1} normal field appliction concentration)	Inhibited N_2O production	Kinney *et al*., 2005
aldrin		10 µg ml^{-1}(similar concentration to field uses) Same to above	Inhibited N_2O emission	Saez *et al*., 2006
lindane				
methidation				
diflubenzuron				
dimetoate				
atrazine				
simazine				

1.3.3 Impact of fertilization on denitrification and N_2O emission

Some of the fertilizers hydrolyzed in soil give an acidic reaction, while others are alkaline forming, leading to the different effect on denitrification. The dissolution of organic matter is affected by alkaline forming fertilizers liquid anhydrous ammonia (LAA), thus increasing the amount of solubilized carbon and nitrogen which can be used for denitrification (Norman et al., 1987). Accordingly, higher emissions of N_2O and N_2 gas after application of alkaline-hydrolyzing fertilizers, such as anhydrous NH_3, urea, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, was observed than after application of acidic fertilizer NH_4NO_3, $NH_4H_2PO_4$ (Mulvaney et al., 1997). However, it is also reported that large amounts of a mixture of different fertilizers could decrease denitrification (Šimek and Hopkins, 1999). Long-term field trial showed that potential denitrification rates were much lower in pots fertilized with ammonium sulfate ($(NH_4)_2SO_4$) compared with calcium nitrate ($Ca(NO_3)_2$) (Enwall et al., 2005). Similarly, the denitrification rates are higher with the application of potassium nitrate (KNO_3) than an ammonium sulfate-based fertilizer in a flooded subtropical soil (Aulakh et al., 2000). However, numerous studies reported that organic fertilizers, such as manures, crop residues, sewage sludge, and composted wastes, promote denitrification more than mineral nitrogen fertilizer (Rochette et al., 2000; Enwall et al., 2005; Dambreville et al., 2006a). The stimulation of denitrification by organic fertilizers is probably due to the provision of available organic carbon.

The application of large amounts of nitrogen fertilizers to agriculture fields influences processes of nitrification and denitrification, and results in increased N_2O production (Akiyama et al., 2006). A series of researches have focused on the effect of fertilizer on denitrification in soil. Nitrogen fertilizers promote denitrification activity in agriculture soil and a large amount of nitrogen is lost through denitrification. The combination of high nitrogen application rates and poor soil drainage can lead to high denitrification activity than lower application rates and good drainage (Hofstra and Bouwman, 2005). Also, pH can be changed by fertilizer both directly and indirectly to affect denitrification activity. In general, denitrificaiton rates

are higher at neutral regions than at acidic regions (Šimek and Cooper, 2002).

Fertilizer can also affect the N_2O/N_2 ration due to denitrificaiton, and N_2O emission is obviously increased due to an increased input of fertilizer (Deklein and Vanlogtestijn, 1994; Kaiser et al., 1998). The application of mineral fertilizer induced higher N_2O losses throughout the crop season compared with the unfertilizerd soil. It has also reported that N_2O emission increased with the amounts of applied manure (Akiyama et al., 2004). Application of poultry manure exhibited higher stimulation of N_2O emission than swine and cattle manure (Dong et al., 2005). Fresh cattle slurry combined with calcium ammonium nitrate mineral fertilizer increased N_2O flux during the first 4 days after application, and it is probably due to the early decomposition of slurry carbon (Dittert et al., 2005). However, the differences of N_2O emission between application of mineral nitrogen fertilizer and slurry were varied with soil type and fertilizer application rates (Grogan et al., 2004).

1.3.4 Natural factors influencing denitrification activity and N_2O emission

Environmental factors influencing N_2O production by denitrifying microorganisms include pH, availabilities of carbon, NO_3^-, NO_2^-, soil moisture, soil pore structure, aeration, temperature, freezing-thawing and drying-wetting events. Because several of these factors influenced by climatic condition uncontrollable, the estimated nitrogen losses are highly variable in time and space.

In some studies, the highest N_2O emission was reported during spring (Parsons et al., 1991; Kaiser and Heinemeyer, 1996), in others during spring and autumn (Ambus and Christensen, 1995), or in summer (Bremner et al., 1980). Soil temperature and soil water content are known factors that affect gaseous nitrogen losses and the N_2O/N_2 ratio. Increasing soil temperature exponentially increased N_2O/N_2 ratio under constant laboratory conditions (Maag and Vinther, 1996). Some studies found a positive correlation between soil temperature and denitrification activity (Bailey, 1976; Keeney et al., 1979), whereas others observed no relationship upon temperature. This might be attributed to the lower water content caused by increased plant transpiration

rates at higher temperatures leading to a water deficiency. Soil water content is linked to oxygen availability. At moisture content between 40% and 60% and 10% oxygen concentration, denitrification is the main source of N_2O production (Lensi et al., 1995). In addition, potential of denitrification were strongly influenced by soil types, as reported to be higher in pasture than in cropped soil (Sotomayor and Rice, 1996).

Early in 1990, Christensen and Tiedje first reported that during thaw periods in spring, arable soils exhibited the most active N_2O emission in an acid sandy loam soil (pH3.8) (Christensen and Tiedje, 1990). Independent from the amount of applied fertilizer, about 70% of the annual N_2O was emitted during winter period (Sehy et al., 2003). The temporal changes of the N_2O emission rates were correlated to dynamics of soil temperature. In contrast, low and stable temperatures below the insulating snow or ice cover decreased N_2O emission. However, some authors showed that N_2O emissions during winter are related to the soil nutrients. Soluble carbon, applied as plant extract, was necessary to induce N_2O production during freezing and thawing event (Christensen and Christensen, 1991). Additionally, the increased concentrations of ammonium and NO_3^- during freezing period were associated with maximum N_2O emission in the following thawing period (Muller et al., 2002). Therefore, the freeze-thaw-induced emission of N_2O could be a straightforward result of enhanced denitrification. However, only few works have been done for the effect of freeze-thaw on denitrifer community composition responsible for N_2O emission. Koponen *et al.* reported that neither microbial biomass nor community structure was affected during freeze-thaw events in boreal soils (Koponen et al., 2006), whereas Eriksson et al. reported a contradictory result as an obvious change of the community structure in ribosomal internal spacer analysis (Eriksson et al., 2001).

Similarly to freeze-thaw cycles in soil, dry-wet cycles can enhance N_2O emission. Comparing the effect of drying-wetting and freezing-thawing events on the emission of N_2O, up to a 1000-fold increase of N_2O emission rates from the cores after wetting or thawing (Prieme and Christensen, 2001). Some studies have also reported that the differences of denitrification between the wet-up and dry-down phases of soil moisture are attributed to rainfall events (Prieme and Christensen, 2001).

1.3.5 Effect of carbon source on denitrifier community

Carbon (C) availability is one of the most important factors to control denitrificaiton rates (Beauchamp et al., 1989). The influence of carbon on denitrification is both through the provision of C directly to the denitrifiers and stimulation of microbial metabolism, which increase the consumption of O_2 and creates conditions favorable for denitrification (Beauchamp et al., 1989). Most studies on the effects of organic C on the denitrification process were conducted under anoxic conditions.

These studies investigated response of soil microorganisms, particularly in microbial activity and microbial abundance. Population size of denitrifiers was quantified in several environmental studies, in which an influence of organic C on emergence of denitrifiers has been reported (Morales et al., 2010). It was also reported that addition of organic matter led to the compositional changes of denitrifier communities as the abundance of certain board groups of denitrifiers among 10 agricultural and other land-use practices at long-term ecological sites (Morales et al., 2010), those after application of fertilizer and organic amendments (Chen et al., 2010), and those after incorporation of crop residues (Miller et al., 2008). However, it was also reported that there were no changes in the compositions of nitrate reducers followed after daily-application of artificial root exudates for one month (Henry et al., 2008). Changes of the targeted communities in population density were related to C availability in soil regardless of C source, as indicated by soil respiration. Previous research examined how various C sources (glucose, plant residues, and liquid manures) influence the response of the soil denitrifier community: the abundance of some components of the soil denitrifier community changed in response to C amendments, while other components remained unchanged (Miller et al., 2008). In their further studies of anoxic soil microcosms, application of C amendments in agricultural soils not only increased microbial activity but also induced changes in total bacterial and denitrifier community structures (Miller et al., 2012).

1.3.6 Nitrous oxide (N_2O) emission from Andisol

Andisol and volcanic ash soils cover about 50% of agricultural fields for consecutive upland crop cultivation in Japan, which was formed by the weathering of volcanic ash and characterized by low hulk density and well-drained aerobic conditions (Ding et al., 2007; Hayakawa et al., 2009).

Because of their high porosity, volcanic ash soils tend to maintain their aerobic conditions and, as much as 87%-92% of the N_2O production is derived from autotrophic nitrification, whereas N_2O emitted after heavy rains (after mid-July) was produced mainly by denitrification (Kusa et al., 2006). Hence, remarkably high N_2O emission rates are (2-6 mg N m^{-2} h^{-1}) probably resulted from the promotion of N_2O production by a relatively high concentration of soil NO_3^- together with a large amount of water added to the soil by heavy rain. Thus, the high N_2O emission rates can occur from poorly drained Japanese agricultural Andisol (Kusa et al., 2006). In previous study, Andisols were also net sinks during unvegetated period and the rations of N_2O emissions to chemical fertilizer N in Japanese Andisols were lower than those in other soils in Japan and around the world (Akiyama and Tsuruta, 2002; Akiyama and Tsuruta, 2003). However, in the present study, the amount of N_2O emission relative to the amount of applied N are markedly higher than those previously reported for Japanese Andisols (Kusa et al., 2006). The large difference in the ration of N_2O emission to chemical fertilizer N between Andisols and gray lowland soil was caused by differences in the physical properties of the soils, such as bulk density and texture (Koga et al., 2004). Vitric Andisol from the Shizunai Experimental Livestock Farm is known as a relatively active N_2O emission (Katayanagi and Hatano, 2005). Hence, it is of great importance to explore biocontrol methods to regulate N_2O emissions of Andisol by N_2O emitters.

1.4 Research outline

1.4.1 Research basis and objective

So far as reported, N_2O-emitting bacteria *Janthinobacterium* sp., *Burkhodleria tropica* and *Burkhodleria cepacia* were directly isolated from agricultural tropical peat soil farms in Central Kalimantan, Indonesia (Hashidoko et al., 2008), one of the most active N_2O emitting sites in the world (Takakai et al., 2006). In addition, three eubacteria *Leptotheix* sp., *Paenibacillus* sp., *and Streptomyces* sp. were isolated as culturable N_2O emitters from the farmland of Andisol located in Hokkaido, Japan, known as a site actively flux N_2O during the spring to summer seasons (Takeda et al., 2012) .

The objectives of this study were to isolate N_2O emitters from fertilized corn farmland and to investigate their biological properties. The isolated N_2O emitters can be used as a model bacterium in studies of practical biological regulation of N_2O production in the temperate agricultural fields.

1.4.2 Research approach and theme

First of all, to obtain N_2O emitters as candidates for bioassay use in the thesis studies on biological regulation of N_2O production, culturable microorganisms (40 isolates) from the rhizosphere of a post-harvest corn farmland in an Andisol collected in autumn, 2011, were screened on MWG plates supplemented with KNO_3 to focus on the process of denitrification. As a result, four isolates exhibited higher N_2O producing activity in the culturing N_2O emission assay. All the active N_2O emitters were identified as Gram-negative bacteria of genus *Pseudomonas*.

Pseudomonas spp. 10CFM5-1B, 10CFM5-2D and 10CFM15-2A that did not show significant acceleration of N_2O production upon exposure to 10% C_2H_2 are considered as incomplete denitrifiers, while the complete denitrifier *Pseudomonas* sp. 10CFM5-2B showed a statistically significant acceleration of N_2O emission. The complete denitrifier *Pseudomonas* sp. 10CFM5-2B and active incomplete denitrifier

Pseudomonas sp. 10CFM5-1B, *Pseudomonas* sp. 10CFM5-2D were selected as test bacteria for N_2O emission assay upon exposure to chemicals (fungicides, herbicides, and a representative secondary metabolite of corn).

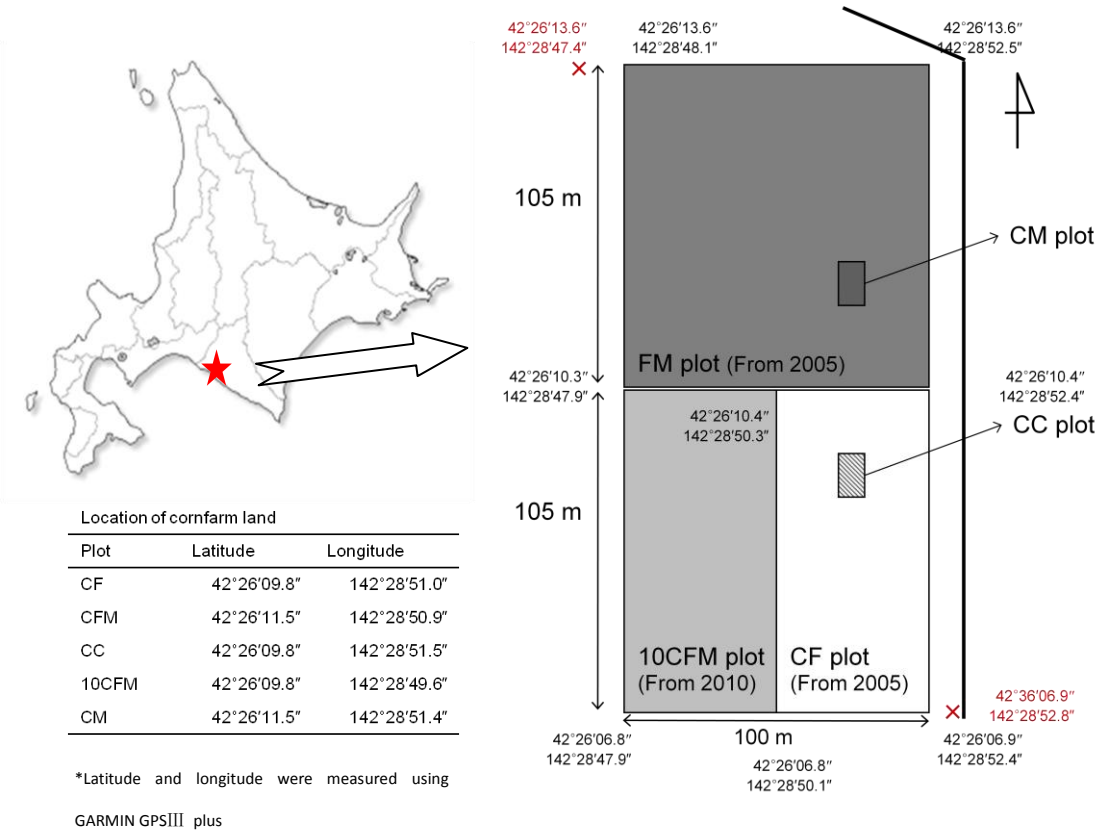
In the first theme of study, we described the isolation and identification of active N_2O emitters, and their characteristics. To investigate the magnitude of N_2O emission by abiotic factors (mainly focused on carbon and nitrogen sources), we described the effects of sucrose and inorganic nitrogen on the diversity of denitrifiers associated with N_2O emission using different methods in the second theme. Firstly, the profile of 16S rRNA gene-targeted DGGE of DNA extracted from Andisoil was investigated. The bacterial succession in the culture of gellan gum medium supplemented with alternative nitrogen and carbon sources would be a model for response of the soil bacteria community to alternative carbon and nitrogen sources. Finally, Andisol culturing assay for N_2O emitters should be the most optimum method for investigating the diversity of nitrifier and denitrifiers.

Chapter 2

Materials and Methods

In this chapter, collection of soil samples from Shizunai Experimental Livestock Farmland; isolation, screening of N_2O emitting ability, incubation, and identification of the culturable microorganisms are described. In addition, optimal conditions of the selected N_2O emiters for N_2O production and quantitative and qualitative methods for bioassays to evaluate N_2O emitting capabilities are described.

2.1 Sampling sites and preparation of soil samples



Location of cornfarm land

Plot	Latitude	Longitude
CF	42°26'09.8"	142°28'51.0"
CFM	42°26'11.5"	142°28'50.9"
CC	42°26'09.8"	142°28'51.5"
10CFM	42°26'09.8"	142°28'49.6"
CM	42°26'11.5"	142°28'51.4"

*Latitude and longitude were measured using GARMIN GPSⅢ plus

Figure 2.1 Location of farmland fields for sampling sites in Shizunai, Hokkaido, Japan.

Soil samples (10 g each) were collected from Shizunai Experimental Livestock Farm, Field Science Center for the Northern Biosphere of Hokkaido University in Southern Hokkaido, Japan (shown as asterisks on the map). The soil samples were collected at following periods: after harvest period in November 2011, prior to fertilization and agitation and after snow melting of the fields.

Soil samples (approximately 10 g) were collected from Shizunai Experimental Livestock Farmland in Hokkaido, Japan after the dent corn was harvest in November 2011 and before fertilization and agitation in April 2012 (Fig. 2.1). With the untreated control of corn field as neither fertilizer nor manure (CC), three treatments applied to the corn field; chemical fertilizer (CF), manure (beef cattle manure with bedding litter) (CM), chemical fertilizer and manure (CFM), all of which were deposited since 2005 to present, and chemical fertilizer and manure deposited since 2010 to the present (10CFM). The application rates of chemical fertilizers were 104 kg N ha^{-1} (80 kg ha^{-1} of ammonium-nitrogen and 24 kg ha^{-1} of urea), 144 kg P_2O_5 ha^{-1} and 80 kg K_2O ha^{-1}. Manure (beef cattle manure with bedding litter) was applied as 215 kg N ha^{-1}, 397 kg P_2O_5 ha^{-1}, and 325 kg K_2O ha^{-1}. At the same time, we also collected soil samples in November 2011 and April 2012 from plots of cultivated pasture located in Shizunai Experimental Livestock Farmland that had been treated with same manner, labeled as CP (control), PF (fertilizer), and PFM (fertilizer and manure). The application rates of fertilizer were 66.1 kg N ha^{-1}, 175 kg P_2O_5 ha^{-1}, and 100 kg K_2O ha^{-1}. Application of the manure were 95.5 kg N ha^{-1}, 74.8 kg P_2O_5 ha^{-1}, and 164.6 kg K_2O ha^{-1}. Soils (48 samples) were obtained in each plot from three points (triplicate) at two different depths, 5 cm (4–6 cm) and 15 cm (14–16 cm), for each season. Samples were obtained before agitation with a rotary cultivator in autumn, 2011 and after snow melting in spring, 2012, then kept in zippered plastic bags at 4 ℃ until use.

2.2 N_2O emission assay for soil suspension under an alternative N-source

To investigate the main causative microorganisms of N_2O production, two media were used alternatively. As mineral N for the substrate of N_2O production, an excessive concentration of $(NH_4)_2SO_4$ (500 mg L^{-1}-N, as 2.4 g L^{-1} of $(NH_4)_2SO_4$) or KNO_3 (500 mg L^{-1}-N, as 3.6 g L^{-1} of KNO_3) was added to Winogradsky's mineral solution separately with 0.01% (w/v) $CaCO_3$ (Hashidoko et al., 2002). The pH of the solution was adjusted to 5.0 with 1 M H_2SO_4, followed by filtration through a

polytetrafluoroethylene (PTFE) membrane (pore size, 0.45 μm) to remove insoluble mineral salts. Gellan gum powder was added as gel matrix to be 0.3% (w/v) in the resulting solution. The mixture was first heated at 117 ℃ for 15 min, and mixed well to completely dissolve the gellan gum. After cooling to room temperature, a 10.0 mL medium was poured into a 30 mL gas-chromatographic vial (Nichiden-Rika Glass Co., Kobe, Japan) to be sealed with a butyl rubber plug and a screw cap septum and then autoclaved at 121 ℃ for 15 min. The headspace volume of the vial that contained 10.0 mL medium has been determined as 22.6 mL (Hara et al., 2009). We further compensated headspace volume as 22.5 mL due to consideration of the inoculant volume (generally 100 μL) unless otherwise mentioned. Soil suspensions were prepared by adding 10 mg fresh soil to 10 mL of sterilized water, vortexing for 1 min in an 18-cm test tube, and standing it for 10 min. For each sample, a 100 μL aliquot of the supernatant was inoculated into an autoclaved 10.0 ml soft gel medium and thoroughly vortexed for 1 min. The vials were incubated in the dark at 20 ℃ for 7 days. The headspace gas in each vial was sampled with a 1 mL gas tight syringe and analyzed with a gas chromatograph (GC) (Shimadzu GC-14B, Kyoto, Japan). The GC equipped with an electron capture detector (ECD) (Shimadzu ECD-2014) kept at 340 ℃ was connected with a 1 m Porapak N column (Waters, Milford, MS, USA) and kept at 60 ℃, using a carrier gas of Ar with 5% CH_4.

2.3 Isolation and screening of N_2O-emitting bacteria

Culture medium from which N_2O can be produced was used according to an N_2O emission assay for the isolation of N_2O-emitting bacteria. A 100 μL aliquot of the suspension (medium diluted 10,000 fold with sterilized water) was inoculated into the modified Winogradsky's gellan gum plates (MWG, Winogradsky's mineral mixture with 0.5% sucrose as the carbon source, 500 mg L^{-1}-N as the nitrogen source, pH 5.0 with 1 M H_2SO_4, and 2% gellan gum for the gel matrix) with a sterile micropipette, and the droplet was spread onto the gellan plate with a spreader. The plates inoculated with the supernatant of the soil suspension were incubated at 20 ℃ in the dark for 4

days. The dominant bacterial colonies apparent on the MWG plates were isolated and purified several times on MWG plates. For investigation of the N_2O emitters, two loops of the isolated bacterial colonies were inoculated into the soft gel medium without supplementation of sugar and vortexed for 30 seconds. All the vials were analyzed by ECD-gas chromatography after incubating at 20 ℃ in the dark for 7 days, at which point the concentration of N_2O in the headspace gas reached its maximum level. When the headspace gas (22.6 mL volume) contained 1 μL L^{-1} of N_2O (1 ppmv, equivalent to 2.0 μg L^{-1}), absolute amount of the N_2O produced from the culture medium per a vial is 45.2 ng. Absolute amount of N_2O in the headspace was simply divided by the incubation days, leading to N_2O emitted per a day from the 10 mL culture (as ng d^{-1} or μg d^{-1}).

2.4 Identification of N_2O emitters

Each bacterial isolate was cultured on a shaker at 110 rpm in 50 mL MW medium at 20 ℃ in the dark for 24 h. Liquid medium was transferred to a sterilized 50 mL Falcon tube and centrifuged at 8000 ×*g* for 10 min to obtain bacterial cells. The bacterial cells were washed with sterilized water several times and finally suspended in 1.5 mL TE buffer. DNA was extracted by using an Isoplant II DNA Extraction Kit (Wako, Osaka, Japan). Using extracted DNA as the template, the genes encoding 16S rRNA were amplified by PCR (TaKaRa PCR Thermal Cycler Dice TP600, Otsu, Japan) with its universal primer pair 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′)/1525R (5′-AAA GGA GGT GAT CCA GCC-3′) (Hashidoko et al., 2008). PCR reactions were done as follows: preheat at 95 ℃ for 5 min, denature with 35 cycles at 95 ℃ for 30 s, anneal at 55 ℃ for 30 s, and extend at 72 ℃ for 30 s, and complete at 72 ℃ for 7 min. The second amplification of sequencing PCR used 3 forward (27F, 341F, 1112F) and 3 reverse (803R, 1080R, 1492R) primers. The conditions for the sequencing PCR were 25 cycles of 30 s at 96 ℃ for denaturation, 15 s at 50 ℃ for annealing, and 4 min at 60 ℃ for extension. The determined sequence was searched in the BLASTN database program provide by the DDBJ (DNA Date

Bank of Japan, National Institute of Genetics, Mishima, Japan) or NCBI (National Center of Biotechnology Information, USA).

2.5 Optimal conditions for N_2O production

To evaluate effect of a supplemented carbon source on N_2O emitters, a series of different concentrations of sucrose (zero, 0.05%, and 0.5%) were added to 10 ml of Winogradsky's medium supplemented with 5 mg KNO_3-N. To obtain fresh inoculates for the N_2O emission assay, *Pseudomonas* sp. was cultured on a shaker at 110 rpm in 50 mL of Winogradsky's medium supplemented with 0.5% sucrose at 20°C for 24 h in dark. Inoculates were collected from 50 mL culture medium by centrifuging at 8000 ×*g* at 4 ℃ for 10 min, washed with Milli-Q water several times, and then dissolved in sterilized water. The bacteria suspension (10^6 cells per milliliter) was added to Winogradsky's medium in 30 mL gas chromatographic vials. After 4 days incubation at 20 ℃ in the dark, the headspace gas was analyzed with GC. The initial medium containing 0.05% of sucrose, from which the N_2O emitters that grew after 7 days incubation, was used for further incubation to analyze optimum pH and N_2O emission.

The pH values of the medium before and after culturing were recorded to check whether any drastic pH changes occurred during incubation of the test bacteria. The optimum pH of the gellan gum medium for active N_2O emission was investigated between pH 3.5 and 7.6. Adjustments in pH were made with 1 M H_2SO_4 and 1 M KOH before autoclaved. The optimal pH was measured by a portable pH meter Horiba F-22 (Horiba, Kyoto, Japan) connected to an Orion 8013BN glass-electrode (Orion, Beverly, MA, USA) cleaned with 70% ethanol. The optimum pH of Winogradsky's gellan gum medium for active N_2O emission was investigated in the range of pH 3.2-6.7 after the culturing.

2.6 Acetylene inhibition assay of N_2O-emitting bacteria

To investigate the effect of acetylene (C_2H_2) on N_2O emitters, N_2O-emitting

bacteria were inoculated into 10 ml soft gel medium in the same gas chromatographic culture vials as used in the N_2O emission assay, and pure C_2H_2 gas (2.25 mL) was injected into headspace (22.57 mL), to give a concentration of 10% C_2H_2 (Balderson et al., 1976). To allow excess gas to escape during the injection of C_2H_2, a sterile needle had been put in place by penetrating the butyl rubber plug. Cultured vials with inoculates but without injection of C_2H_2 gas were prepared as controls. Treated samples and controls were examined in triplicate. After incubation for 5-7 days, the amount of N_2O in the headspace gas was measured by ECD-gas chromatography (Shimadzu GC-14B equipped with an ECD detector, Shimadzu ECD-2014) as described above.

2.7 Detection of *nosZ* gene from N_2O emitting *Pseudomonas* spp. by PCR

In denitrifying pseudomonads, detection of *nosZ* was done by PCR, using a primer pair nosZ-1111F (5′-STA CAA CWC GGA RAA SG-3′) and nosZ-1773R (5′-ATR TCG ATC ARC TGB TCG TT-3′) (Scala et al., 1998). PCR conditions were as follows: preheating at 95^{o}C for 5 min, 35 cycles of denaturation at 95^{o}C for 30 s, annealing at 50^{o}C for 30 s, and extension at 72^{o}C for 30 s. Finally, the reaction was completed at 72^{o}C for 7 min. Annealing temperatures were set at relatively high at 50^{o}C, allowing no emergence of non-specific PCR amplicons. Sequencing of the PCR amplicons assignable as *nosZ* fragments by agarose gel electrophoresis was attempted. If one bacterium gave a single amplicon, its sequence determination followed by the homology search on DNA database (NCBI) was done to confirm whether the bacterium possesses *nosZ* gene in its genome. For the quality check of the template DNAs obtained from the bacteria that were subjected to the *nosZ* PCR assay, 16S rRNA gene region was also amplified with a 16S rRNA gene-targeted universal primer pair 27F/1525R as the reference PCR products.

2.8 N_2O emission assay for soil in gas-chromatographic vials

A portion (5 g) of raw soil collected at depth of 5 cm from the experimental farm was placed in a gas-chromatographic vial with a butyl-rubber cap (Nichiden-Rika Glass Co., Kobe, Japan). As a nitrogen source, 500 μL of nitrate or ammonium solution (5 mM KNO_3, 5 mM NH_4NO_3, or 2.5 mM $(NH_4)_2SO_4$) was added to the soil-containing vial at a final concentration of 0.1 mM (0.1 μmol g^{-1} soil). For the vials that were further supplemented with a carbon source, sucrose was added to each nitrate or ammonium solution at a concentration of sucrose was 0.5 mg g^{-1} soil (~1.5 mM). All soil samples were incubated at 20℃ for 5 days in the dark. Dormant seeds of weeds from the soil often germinated during the incubation, but those were not removed from the soil. N_2O in the headspace gas was quantified using ECD-gas chromatography after the incubation time. The quantitative analysis was carried out as described in a previous study (Takeda et al., 2012).

Table 2.1 Winogradsky's medium supplemented with alternative C- and N-sources.

Treatment	Sucrose ($g\ L^{-1}$)	KNO_3 ($g\ L^{-1}$)	$NH_4\ NO_3$ ($g\ L^{-1}$)	$(NH_4)_2\ SO_4$ ($g\ L^{-1}$)
Control	--	--	--	--
Sucrose	5	--	--	--
Sucrose+ KNO_3	5	0.5 (5 mM)	--	--
Sucrose+$NH_4\ NO_3$	5	--	0.4 (5 mM)	--
Sucrose+ $(NH_4)_2\ SO_4$	5	--	--	0.3 (2.5 mM)
KNO_3	--	0.5 (5 mM)	--	--
$NH_4\ NO_3$	--		0.4 (5 mM)	
$(NH_4)_2\ SO_4$	--			0.3 (2.5 mM)

--, without supplements

2.9 N_2O emission assay for incubation of soil suspension with optional carbon and nitrogen source

The medium supplemented with optional nitrogen and carbon sources was shown in Table 2.2.

Table 2.2 Winogradsky's medium supplemented with optimal carbon and nitrogen sources.

Treatments	Sucrose ($g\ L^{-1}$)	KNO_3 ($g\ L^{-1}$)
Control	--	--
KNO_3+Sucrose	0.5	0.36 (3.6 mM)
KNO_3	--	0.36 (3.6 mM)
Sucrose	0.5	--

--, without supplements

Soil suspensions were prepared from 10CFM, by adding 10 mg fresh soil to 10 mL of sterilized water, vortexing for 1 min, and standing for 10 min. For each medium, a 100 μL aliquot of the supernatant was inoculated into 10.0 ml soft gel medium (Table 2.2) and vortexed for 1 min. The vials were incubated in the dark at 20 ℃ for 7 days in the dark. The headspace gas in each vial was sampled with a 1 mL gas-tight syringe and analyzed with a gas chromatograph (Shimadzu GC-14B equipped with an ECD detector (Shimadzu ECD-2014), Kyoto, Japan).

2.10 N_2O emission assay for soil cultured in medium with alternative carbon and nitrogen source

To analyze the N_2O emission potentials and microbial diversities of bacteria consortia under different N-source substrate, KNO_3 (5 mM), NH_4NO_3 (5 mM), $(NH_4)_2SO_4$ (2.5 mM) as sources of mineral N were added to the Winogradsky's mineral medium separately as shown in Table 2.1. In order to observe the effect of carbon sources on N_2O emission, the medium supplemented with 0.05% sucrose was also used (Hashidoko et al., 2008). The pH of the medium was adjusted to 5.0 with 2M H_2SO_4, filtered through a hydrophilic 0.45 μm PTFE membrane, and then solidified with 0.3% (w/v) gellan gum (Wako Pure Chemical Industries, Osaka, Japan). The medium was autoclaved at 117℃ for 15 min until gel powers were evenly dissolved. After it was cooled to room temperature, 10 ml medium was injected to a 30-ml gas-chromatographic vial (Nichiden-Rika Glass Co., Kobe, Japan), sealed with a butyl rubber plug with a screw cap septum, and then autoclaved at 121℃ for 15 min.

A portion of 10 mg fresh Andisol from 10CFM was directly added to the 10-ml medium, vortexed for 1 min to thoroughly mixed, and then incubated at 20℃ for 5 days in dark. Each treatment was in triplicate. The headspace volume of the plugged vials was exactly 22.5 ml (Hara et al., 2009) and was used to calculate the concentration of produced N_2O production in each vial.

2.11 DNA extraction from soil

A 5 g portion of soil sample, both collected in autumn, 2011 and Spring, 2012, at two depth as of 5 cm and 15cm was separately added to a 50-ml falcon tube contained glass beads. A 9.5 ml of Lysis solution BB and 0.5 ml of Lysis solution 20S were added to the tube, and then vortexed at the highest speed (14,000 ×g) for 10 min. Then, the tubes were incubated at 65℃ for 60 min, vortexed every 10 min-interval. The tubes were centrifuged at 4,000 ×g for 5 min to remove the precipitates. The

supernatant (6 ml) was transferred into a new 50 ml tube and 4 ml of Purification Solution was added and mixed them well, followed by addition of 6 ml $CHCl_3$ to the supernatant, vortexed for 1 min, and then centrifuged at 4,000 ×g for 15 min. The resulting aqueous layer (8 ml) was transferred to a new centrifuge tube (50 ml) without contamination of the intermediate layer. An equal volume of the Precipitation Solution was added, mixed well, and centrifuged at 8,000 ×g for 60 min at 4℃. After the supernatant was discarded, the precipitates on the bottom of the centrifuge tube was rinsed with 5 ml of Wash Solution, and then centrifuged at 8,000 ×g for 10 min at 4℃. The supernatant was discarded and added 5 ml of 70% EtOH and 10 μl of Ethachinmate, a DNA co-precipitating reagent, vortexed, and then centrifuged at 8,000 ×g for 10 min at 4℃. After the supernatant was removed, precipitates were air-dried and then dissolved in 30 μl of TE buffer (pH 8.0).

2.12 Extraction of community DNA from medium inoculated with soil suspension

To obtain DNA from bacterial communities, the medium that incubated for soil suspension of 10CFM soil (corn field, fertilizer and manure were applied since spring of 2010) and control soil (without any fertilization) collected in two seasons was prepared. Total 0.9 ml medium (3×0.3 ml of each replicate) was transferred to 2-ml Eppendorf tubes filled with zirconia beads and then cooled with liquid nitrogen. The cells in the medium were crushed using a Multi-Beads Shocker (Yasui- kikai, Osaka, Japan) at 3,000 rpm for 2 min in triplicate. Community DNA was extracted from the disrupted cells using an Isoil DNA Isolation kit (Nippon Gene, Toyama, Japan).

2.13 Amplification of 16S rRNA

The 16S rRNA gene-targeted PCR-DGGE to determine bacterial community structure in the soil inoculated culture was performed using semi-nested PCR with a Mastercycler Gradient (Eppendorf, Hamburg, Germany) and PrimeSTAR HS DNA

Polymerase (Cat # R010A; Takara, Otsu, Japan) using the soil DNA as the template. The primer pairs 341F (5'-CCT ACG GGA GGC AGC AG-3') and 1401R (5'-ACG GGC GGT GTG TAC-3'), and 341F with GC clamp (341gcF, 5'-CGC CCG CCG CGC CCC GGG GTC CCG CCG CCC CCG CCC GCC T AC GGG AGG CAG CAG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') were used for the first and the second PCR respectively (Muyzer et al., 1993; Amann et al., 1995). The reaction mixture was prepared with the template DNA in a final volume of 20 μL. The PCR program was as follows-25 cycles of denaturation at 98 ℃ for 10 s, annealing at 55 ℃ for 5 s, and extension at 72 ℃ for 1 min, followed by a final cooling at 4 ℃. The GC-clamped amplicons (5 μL) were loaded on 1.5% (w/v) agarose gel and analyzed by electrophoresis. The PCR products were purified with a commercial DNA purification kit, MonoFas (Cat # 5010-21502, GL Science, Tokyo, Japan) to be used as a template in the second PCR having the same program as the first PCR.

2.13 DGGE analysis

The DGGE was carried out using a DCode^{TM} Universal Mutation Detection System instrument according to manufacturer's instructions (Bio-Rad). The PCR products were run on polyacrylamide gel (6% w/v) containing a liner formamide/ urea ranging from 30-70% denaturant. The 100% denaturant solution contains 7 mol l^{-1} urea, 40% (v⁄v) formamide, 6% acrylamide/bis-acrylamide (37.5:1) and 0.5×TAE buffer (pH 8) in Milli-Q water. A 10 μL portion of purified PCR products mixed with 10 μl loading buffer were transferred to the bottom of the wells. The gel plate was run at 60 ℃ for 16 h at 160 V (Heuer et al., 1997; Tzeneva et al., 2008; Xiao et al., 2009). Followed by staining with SYBR® Green Ⅱ (Cat # 50523-F0523; Takara) for 30 min. The bands were visualized with a gel scanner, Typhoon Trio (GE Healthcare, Little Chalfont, UK).

2.14 Chemicals used in N_2O emission assay

Six compounds were used in this study. The author obtained 6-methoxy-2-benzoxazolinone (MBOA), 2-benzoxazolinone (BOA), and 1-hydroxy-1*H*-benzotriazole (HOBt) from Wako (Osaka, Japan). *N*-heterocyclic herbicides, methyl viologen dichloride (Paraquat®; reagent grade), simazine (reagent grade), and amitorol (reagent grade) were also purchased from Wako. MBOA is an allelochemical of corn, which shows antifungal and herbicidal activities (Glenn et al., 2001; Rosenblueth and Martinez-Romero, 2004), while both BOA and HOBt are commercially available chemical reagents. BOA is often used as an oxidative-stress inducer (Batish et al., 2006), and HOBt is a redox inhibitor and a coupling reagent for amide synthesis (Hirai et al., 2006).

In a preliminary test, 10 μM of each test compound was exposed to N_2O-emitting bacteria that had been isolated from Andisol corn farmland (see the following subsection). Test compounds that showed an active repression of N_2O emission at 10 μM were further investigated at lower concentrations ranging from 2.5 to 10 μM. Chemicals that showed accelerating activity toward N_2O production of the denitrifier were tested using a culturing assay at 2 and 10 μM concentrations.

2.15 Preparation of the test medium

The initial herbicide used in the present study was methyl viologen dichloride, an electron transport inhibitor known commercially as Paraquat®. Methyl viologen dichloride was dissolved in sterilized water to 1.0 M, and then further diluted with sterilized water to a concentration of 10 mM (100-fold dilution). Ten μl of each diluted solution was added aseptically to the bioassay medium, which was supplemented with 0.05% sucrose, autoclaved at 121 ℃ for 15 min, and then cooled to room temperature. The final concentration of the test medium was 0.1–5 μM. At the same time, chemical-free medium was prepared as the control. Cell suspensions of two N_2O-emittable bacteria (100 μl; 10^6 CFU mL^{-1}) was inoculated to the test and

control media, vortexed, and then incubated at 20 ℃ in the dark for 7 days. Each assay was performed in triplicate.

For other test compounds, a 1 M solution of the compound in dimethylsulfoxide (DMSO) was diluted with sterile water to the desired concentration. For example, a 50 μL of compound solution was again diluted with 450 μL of sterile water to get the 100 mM compound solution. Similarly, a 5 μL of 1 M compound solution was diluted in 495 μL of sterile water and the final concentration was 10 mM. We then added 10 μL of the diluted solution to 10 mL of the medium under aseptic conditions to prepare 1000-fold diluted medium. Subsequent procedures were the same as those performed in methyl viologen dichloride, including incubation and gas analysis. Each treatment was performed in triplicate.

2.16 Culture of N_2O-emittable bacteria and measurement of N_2O

For the N_2O production assay, Winogradsky's mineral solution containing 0.05% sucrose (0.5 g Ł$^{-1}$) and KNO_3 (500 mg Ł$^{-1}$-N, as 3.6 g Ł$^{-1}$ KNO_3) as the carbon and nitrogen sources, respectively, was prepared; 0.3% gellan gum was added as the gelling agent before pre-heating. Ten mL of the medium was poured into a 30-mL gas chromatography vial (Nichiden-Rika Glass Co., Kobe, Japan) and autoclaved at 121 ℃ for 15 min. After the liquefied medium was cooled and gelled again, a loop of *Pseudomonas* sp. 10CFM5-1B or 10CFM5-2D was inoculated into the medium and allowed incubation at 20 ℃ in the dark for 7 days. In the cultured medium, NO_3^- is utilized as an electron donor for nitrate respiration, leading to N_2O production (Hashidoko et al., 2008).

After the incubation, N_2O in the headspace gas was analyzed quantitatively by using ECD (electron capture detector)-gas chromatography (Shimadzu GC-14B, Kyoto, Japan) connected with column (1-m Porapak N column; Waters, Milford, MS, USA) was kept at 60 ℃ by using a carrier gas of Ar with 5% CH_4. A portion of headspace gas (from 50 μL to 1.0 mL) in the vials (22.5 mL) was analyzed by gas chromatography.

2.17 Raising seedlings of dent corn

Seeds of dent corn were imbibed with running tap water and then incubated in distilled water at 25℃ for 2 days until germination. The seeds at an early stage of germination were selected and planted in a 25×35 cm^2 plastic tray containing autoclaved (121℃, 20 min) river sands (approximately 2 L) in plant growth chamber (25℃, the condition of chamber, 16 h light). After 7 days cultivation, corn roots were gently recovered from the soil and rinsed in water.

2.18 Collection and chromatographic analysis of corn roots extracts

After cultivation for 7 days, corn roots were gently harvested from the river sands and rinsed them with deionized water, and then placed in a 200 mL beaker. The corn roots were socked in 100 ml EtOAc at 4℃ for 5 days in dark. The root extract was filtered through filter paper (Advantec, Tokoy, Japan). This extraction was repeated twice.

The organic layer of root extract was combined, dried with anhydrous Na_2SO_4, and then evaporated to dryness under a low pressure at 35℃ using a rotary evaporator (type Buchi, V-850, Flawil, Switzerland). The concentrated (354 mg) were resuspended in hexane-EtOAc (95:5, v/v) and fractionated by chromatography on a silica gel GF_{60} column (50 g, 25 cm × 3 cm, 35-to-70 mesh; Merck) eluted with 220 ml each of 10, 20, 30, 40, 50, 60, 70, 80, 90% EtOAc in n-hexane and 100% EtOAc. Each fraction was concentrated to dryness and re-dissolved in 1 ml MeOH. For semiquantitation, 5 μL of the organic layer was applied to a Kieselgel 60 GF_{254} silica gel thin-layer chromatographic (TLC) plate (0.25 mm, Merck, Darmstadt, Germany) using a volumetric glass capillary tube, then developed in hexane-EtOAc (1:1, v/v). The developed TLC plate was sprayed with vanillin-H_2SO_4 reagent for detection. Totally 22 fractions of 10 ml each were collected. All the fractions were dried up using a rotary evaporator at 35℃ and the roots residues were resuspended in 3 mL of n-hexane. A portion of 100 μL of each fraction was dried again under low pressure at

35 ℃ with a rotary evaporator and dissolved in appropriate volume of MeOH according to the amount of each fraction to make the final concentration 1 mg/ml. 100 μl of each fractions were used to biocontrol assay.

2.19 Biocontrol assay

The seeds were placed in 5 mL of 1% NaCl solution and sterilized with 70% ethanol for 2 mim and then surface sterilized with 2% NaClO for 15 min and washed with sterilized distilled water. The surface-sterilized seeds were transferred to a petri dish containing 10 mL sterilized water.

Corn seeds were incubated at 25 ℃ for 2 days until germination. We selected seeds at an early stage of germination, transferred onto 10 mL of Winogradsky's medium solidified with 0.3% gellan gum in a 30-mL vial, in which 0.3% NH_4NO_3 was added as substrate for N_2O emission. For corn seeds, each vial contains one seed. N_2O emittable bacteria *Pseudumonas* 10CFM5-2B was inoculated to the medium until the roots spread wildly and the leaves grow out of the medium after 1-2 days growth. Treatments were: (a) control (inoculated with *Pseudumonas* 10CFM5-2B); (b) only incubated with seeds; (c) seeds inoculated with *Pseudumonas* 10CFM5-2B. Five replications were made. The vials were covered with aluminum foil in the bottom to keep the roots dark. All the vials were kept in 25 ℃ in growth chamber and 12 h light-darkness for 7 days. At the same time, hairy vetch, sorghum and hey oats seeds were also tested using the same methods, but for these three plants, each vial contains three seeds.

Pseudomonas spp. isolated from Andisol in corn farmland, were identified and characterized as the most active N_2O emitting bacteria. All the strains was preserved in 10% glycerol solution and kept at -80 ℃, routinely grown on MWG plate. To obtain fresh bacteria for bioassay, the active N_2O emitters were shake-cultured in Winogradsky's liquid medium when the strains were kept in their exponential growth stage. Liquid medium subjected to culturing was collected and centrifuged at 8,000 ×*g* for 10 min at 4 ℃, washed with Milli-Q water several times and then resuspended

into sterilized water. Winogradsky's medium solidified with gellan gum was inoculated with 100 μL of bacterial cell suspension (10^6 CFU ml^{-1}). Simultaneously, 100 μL of each fraction was added to the medium to reach the final concentration at 10 mg L^{-1}. Fraction 13 that showed the significant acceleration of N_2O production was selected for identification of active principle from dent corn seeding roots and for further bioassay.

Chapter 3

Results

In this chapter, the results of the experiments including the isolation and identification of active N_2O emitters from Andisol, and their physiological characteristics, such as optimal pH, response of the bacteria to carbon source, and effect of roots extracts and herbicide on N_2O emission, are described.

3.1 Isolation and characterization of nitrous oxide (N_2O)-emitting *Pseudomonas* denitrifiers isolated from post-harvest soil of dent corn Andisol farmland in the Shizunai Experimental Livestock Farm in Hokkaido

3.1.1 N_2O emission capacity of Andisol suspension and N_2O emitters isolated from the farm soils in autumn

The N_2O-emitting capacity of the post-harvest Andisol-farmland soil samples was investigated using the N_2O emission assay done in a gellan gum soft gel medium. Of the 48 Andisol samples that were combined with an alternative mineral nitrogen source (NH_4^+-N or NO_3^--N), 10 soil inoculants showed active N_2O emission greater than 64 ng d^{-1} in the headspace gas in medium (equivalent to 10 ppmv N_2O in the headspace gas after 7-day-incubation) with added NO_3^--N (Fig. 3.1A). All the active cultures were from the corn farm soils. The suspension of soil 10CFM5 (fertilizer and manure applied since 2010, depth of 5 cm) after the incubation exhibited significant N_2O production (122-520 ng d^{-1}), whereas no obvious N_2O production was observed in unfertilized soil (CC and CP) (Fig. 3.1A). Unlike NO_3^--N that led to active N_2O emission from some soils in the bioassay, none of the soil samples demonstrated substantial N_2O emissions when the medium contained NH_4^+-N (Fig. 3.1B), indicating that the major process of N_2O production was nitrate-reducing

denitrification rather than ammonia-oxidizing nitrification (Butterbach-Bahl and Dannenmann, 2011).

From the ten soft gel cultures that showed the most active N_2O emission (* in Fig. 3.1), the microorganisms were isolated as distinguishable colonies spread on MWG plates. Subsequently, microorganisms from the actively N_2O producing cultures were screened and 40 culturable microbial isolates were obtained. N_2O emission potential was investigated for each bacterial or fungal isolate. Four of the 40 isolates exhibited higher N_2O producing activity than 0.3 μg d^{-1} level (Fig. 3.2). All the active bacterial isolates were tentatively identified as Gram-negative bacteria of the genus *Pseudomonas* (Table 3.1). Compared to the N_2O emitters isolated from spring soil of the same Andisol farm collected in April, 2008 (Takeda et al., 2012), the N_2O emittable bacteria screened from the post-harvest soil in autumn showed a relatively high N_2O productivity (Fig. 3.1A).

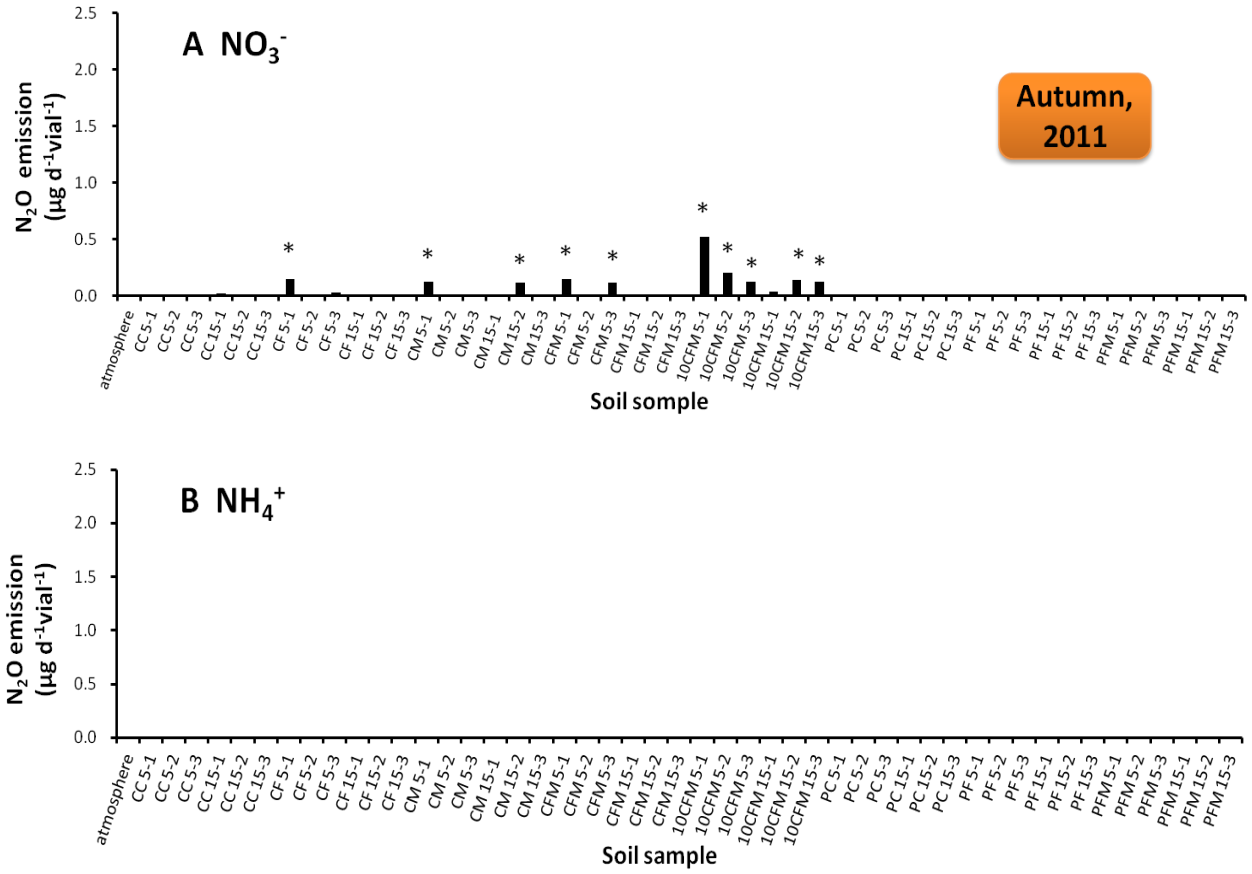


Figure 3.1 N_2O emission potentials of Andisol post-harvest farm soils in autumn sub-cultured in gellan gum soft gel medium.

N_2O emission from soil suspensions incubated in gellan gum soft-gel medium (pH 5.0) with 500 mg L^{-1} NO_3^--N (A) or 500 mg L^{-1} NH_4^+-N (B) at 20 ℃ for 7 days in the dark. Soil samples were collected in Shizunai Experimental Livestock Farmland with different fertilizer and manure treatments.

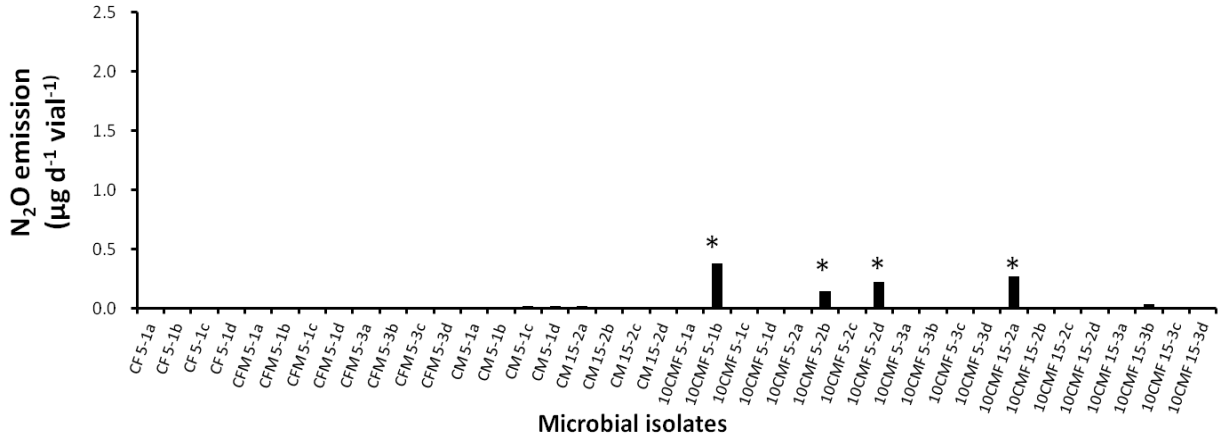


Figure 3.2 N_2O emittable bacteria isolated from Andisol in post-harvest soil suspension inoculated on MWG plates.

MWG plates are Winogradsky's mineral mixture with 0.5% sucrose as the carbon source, 3.6 mg ml^{-1} KNO_3 as the nitrogen source, pH 5.0 with 1 M H_2SO_4, and 2% gellan gum for gel matrix. Two loops of bacterial colonies scraped from a pre-cultured plate were directly inoculated into the soft gel medium (pH=5.0) supplemented with 3.6 mgL^{-1} KNO_3 and incubated at 20 ℃ for 7 days in dark. Arrows indicate the bacteria that were subjected to 16S rRNA gene sequencing as a means for the identification of N_2O-emitters.

Table 3.1 N_2O emitting bacteria isolated from Andisol in autumn 2011.

Isolated strain	Temporal identification	16S rRNA gene (bp)	Accession no.	* N_2O emission ($\mu g\ d^{-1}\ vial^{-1}$)	Type of denitrification (10% C_2H_2 test)
10CFM5-1B	*Pseudomonas* sp.	1377	AB856847	0.38	incomplete
10CFM5-2D	*Pseudomonas* sp.	1416	AB856848	0.23	incomplete
10CFM15-2A	*Pseudomonas* sp.	1417	AB856849	0.27	Incomplete
10CFM5-2B	*Pseudomonas rhodesiae*	1328	AB856850	0.15	complete

Four bacterial isolates were each identified by DNA sequencing and listed as accession number deposited in DDBJ.

***** N_2O emission was analyses in the medium without carbon source.

3.1.2 N_2O emission activity of Andisol collected in pre-sawing spring, 2012

To re-examine the higher N_2O production from spring soil bacterial communities than the post-harvest soil, the author sampled the same corn farmland soils in April 2012 and assessed them by the culturing N_2O emission assay (Fig. 3.3). Nine soil inoculants from soil samples which collected in autumn combined with alternative mineral nitrogen source (NH_4^+-N or NO_3^--N) showed active N_2O emission greater than 0.3 μg d^{-1} in the presence of NO_3^--N. On the contrary, there is no sample demonstrated significant N_2O emission when the soil suspension was exposed to the medium containing NH_4^+-N (Fig. 3.3B). Supernatants of the soil suspensions both from corn farmland and pasture soils collected in spring, 2012 were inoculated to the sugarless culture medium, the similar results were observed compared to the soil collected in autumn (Fig. 3.1). Compared to corn farmland, pasture soil showed the lower N_2O emission resulting in no more than 0.25 ng d^{-1} $vial^{-1}$collected from both spring and autumn (Figs. 3.1 and 3.3). In contrast, cultures from corn farm soils exhibited more active N_2O production. The highest activity was observed on 10CFM5 (fertilizer and manure applied in 2010, depth of 5 cm) while there is no significant N_2O production in unfertilized soil (Fig. 3.3A).

As shown in Fig. 3.4, six bacteria isolated from the Andisol collected in spring showed active N_2O emission performance. All of these active N_2O emitters were identified as *Pseudomonas* sp. The N_2O emission capacity of the most active N_2O emitter *Pseudomonas* 10CFM 5-4A isolated from the soil treated with fertilizer and manure from 2010, were 5 fold higher than *P. chlorophis* 10CFM 5-1B isolated in autumn. These bacterial species which showed a high N_2O emission capacity compared to N_2O emitters isolated in autumn may contribute to the variations of N_2O emission in different seasons.

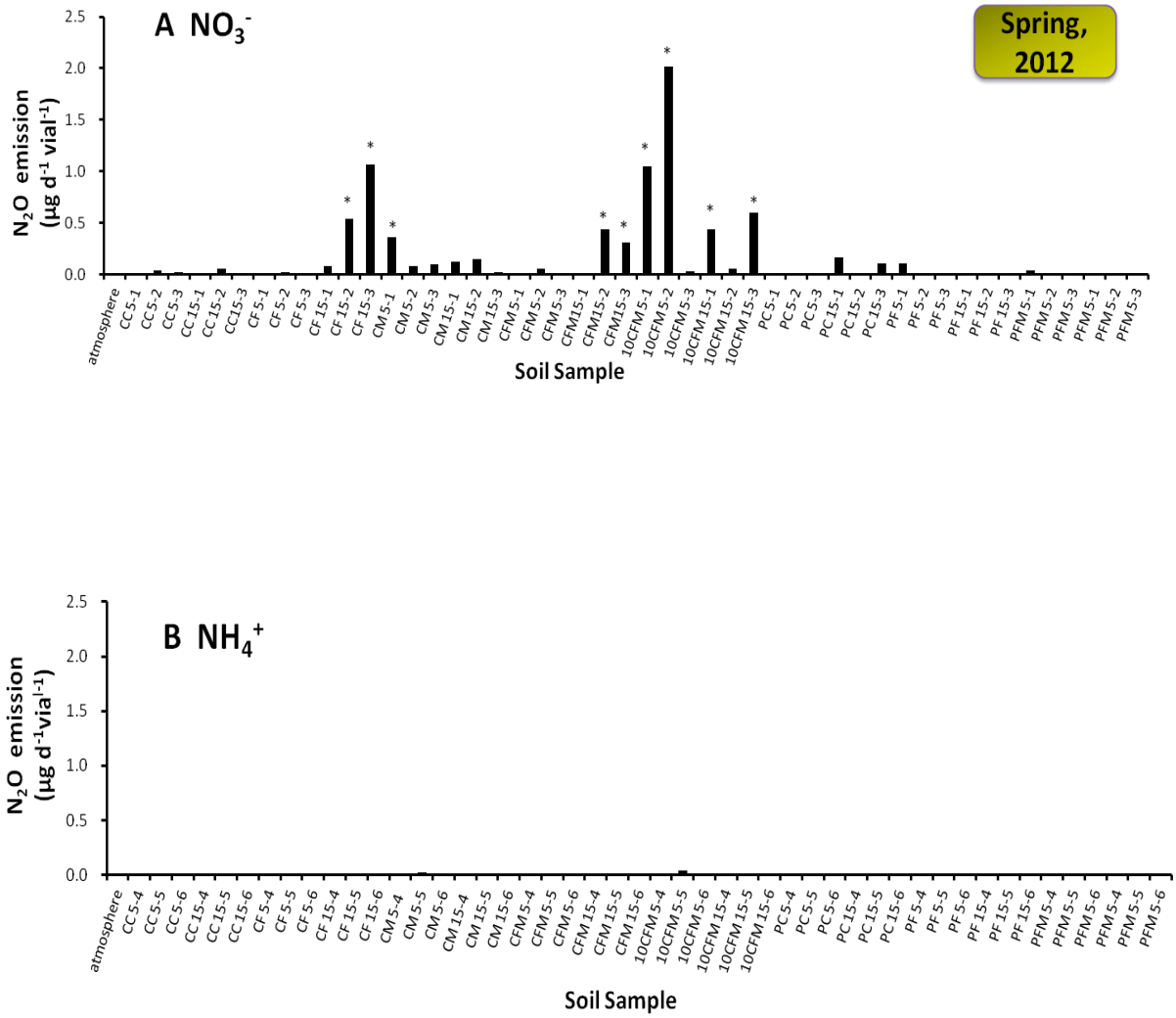


Figure 3.3 N_2O emission potentials of Andisol post-harvest farm soils in spring sub-cultured in gellan gum soft gel medium.

N_2O emission from soil suspensions incubated in gellan gum soft-gel medium (pH 5.0) with 500 mg L^{-1} NO_3^--N (A) or 500 mg L^{-1} NH_4^+-N (B) at 20℃ for 7 days in the dark. Soil samples were collected in Shizunai Experimental Livestock Farm with different fertilizer and manure treatments. * used for further isolation of N_2O emitters.

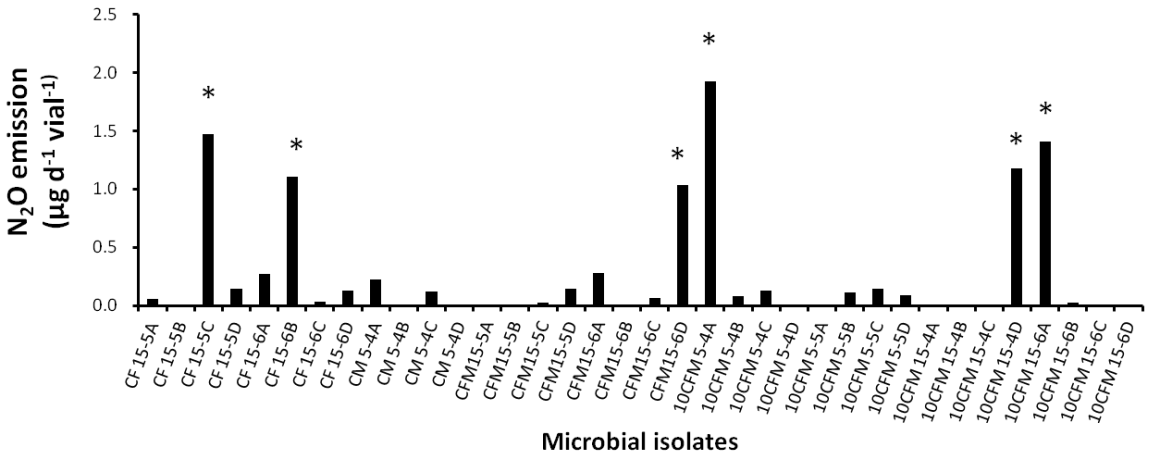


Figure 3.4 N_2O emission bacteria isolated from Andisol in autumn purified on MWG plates.

The condition of medium and plates were same to Fig.3.2.

Table 3.2 N_2O emitting bacteria isolated from Andisol in spring 2012.

Isolated strain	Temporal identification	16S rRNA gene (bp)	Accession no.	Homology (%)	* N_2O emission (μg d^{-1} $vial^{-1}$)	Type of denitrification (10% C_2H_2 test)
CF15-5C	*Pseudomonas* sp.	1465	NR_117821.1	97%	1.47	complete
CF15-6B	*Pseudomonas* sp.	1447	NR 074834.1	98%	1.11	complete
CFM15-6D	*Pseudomonas* sp.	819	NR_024911.1	99%	1.04	incomplete
10CFM5-4A	*Pseudomonas* sp.	1424	NR_116700.1	92%	1.93	complete
10CFM15-4D	*Pseudomonas* sp.	1440	NR_102835.1	99%	1.18	Incomplete
10CFM15-6A	*Pseudomonas* sp.	1467	NR_102835.1	99%	1.41	incomplete

***** N_2O emission was analyses in the medium without carbon source.

3.1.3 Characteristics of N_2O-emitting bacteria *Pseudomonas* sp. isolated from Andisol in autumn

Addition of 0.05% or 0.5% sucrose as a carbon source to the standard assay medium led to increased N_2O production by the N_2O emitters, *Pseudomonas* spp. 10CFM5-1B and 10CFM5-2D (Fig. 3.5). N_2O production from the culture medium was not significant (less than 16 ng d^{-1}) when cultured in the sugarless medium, but N_2O production was approximately 20 times higher in the presence of 0.05% sucrose. N_2O emission from *Pseudomonas* sp. 10CFM5-1B cultured in 0.5% sucrose-supplemented medium produced 36.6 μg N_2O d^{-1} in headspace was more than 4 fold higher than that incubated in 0.05% sucrose-supplemented medium, which suggesting that the carbon source is an important factor for N_2O emission during the denitrification process in *Pseudomonas* spp. 10CFM5-1B and 10CFM5-2D. For further investigation, 0.05% sucrose was selected as the condition for the culturing assay because excessive carbon sources are too far from soil conditions experienced by the N_2O-emitters in the post-harvest soil.

Addition of 10% C_2H_2 did not show any significant acceleration of N_2O production by N_2O-emitting *Pseudomonas* spp. 10CFM5-1B, 10CFM5-2D, and 10CFM15-2A (Fig. 3.6), hence, they are considered to be incomplete denitrifiers. In contrast, *Pseudomonas* sp. 10CFM5-2B demonstrated a significant increase of N_2O emission in the presence of headspace C_2H_2 (4.2 μg d^{-1} with 10% C_2H_2 compared to 2.1 μg d^{-1} in control). In the PCR assay for detection of *nosZ* gene among those four pseudomonads examined, none of the PCR product was detected in the incomplete denitrifier *Pseudomonas* spp., negative of the acetylene inhibition assay. Conversely, only strain 10CFM5-2B was uniquely positive to give an amplicon of 500-600 bp, and its sequence determination followed by the homology search on DNA database (NCBI) confirmed it as a *nosZ* fragment (Fig. 3.7).

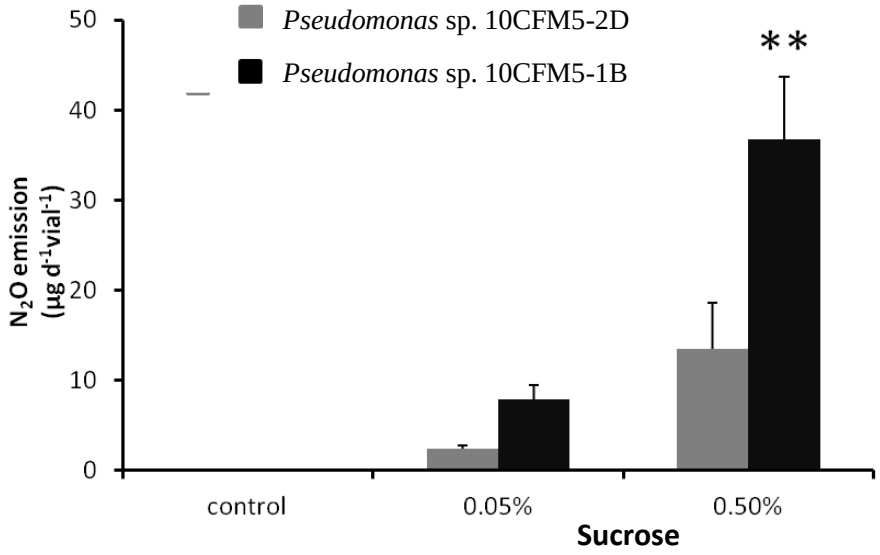


Figure 3.5 Effect of supplemental sucrose on N_2O emission of N_2O-emitting *Pseudomonas* sp.

Effect of supplemental sucrose (0.05%, 0.5%) in Winogradsky's gellan gum medium containing 3.6 mg ml^{-1} KNO_3 on N_2O emission of N_2O-emitting *Pseudomonas* sp. incubated at 20 ℃ for 4 days in the dark. Values are means ± standard deviations (SD, n=3) shown by error bars. ** $P< 0.01$ by Student's-*t* test.

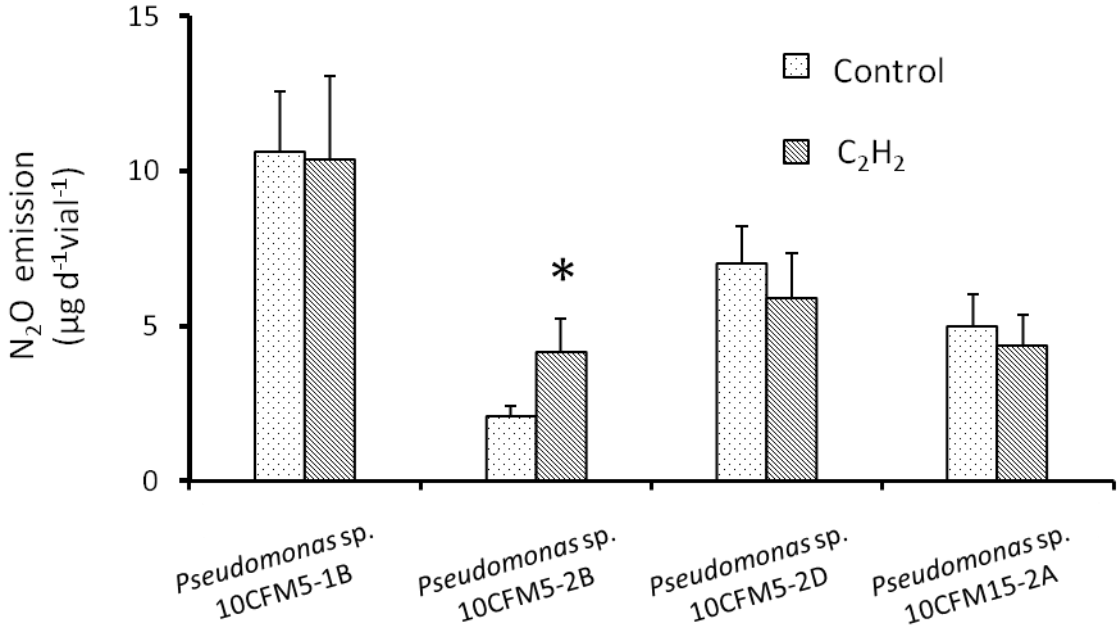


Figure 3.6 Acetylene inhibition assay injected with 10% C_2H_2 gas in the headspace of culture vials for N_2O-emitting *Pseudomonas* spp.

N_2O production of N_2O-emitting *Pseudomonas* spp. in Winogradsky's gellan gum medium supplemented with 3.6 mg ml^{-1} KNO_3 and 0.05% sucrose with the presence of absence of 10% C_2H_2 incubated at 20 ℃ for 4 days in the dark. Values are means ± SD (shown by error bars, n=3). * $P< 0.05$ by Student's-*t* test.

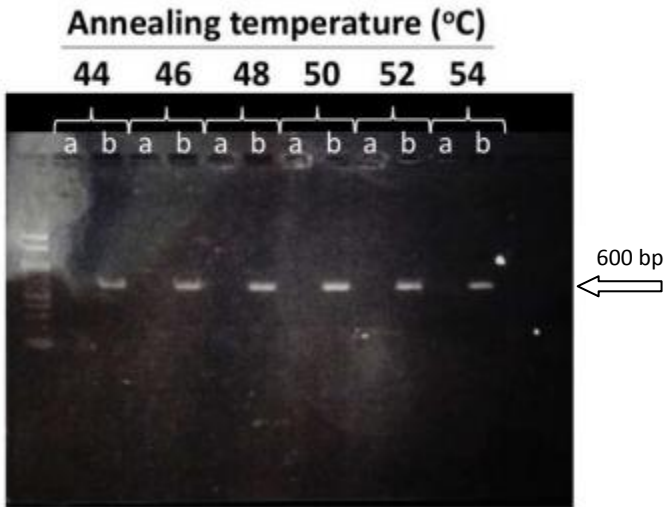
nosZ-1111F

5’-STACAACWCGGARAASG-3’

nosZ-1773R

5’-ATRTCGATCARCTGBTCGTT-3’

a, b, *Pseudomonas* sp. 10CFM5-2B



nosZ gene sequence (564bp)

ACTGGGTGGTGGTGTTCACATTCCGCGTATCGAGGCCGCGATCATGGGCCGGCAAGTTCATC
CACCTGGACGGCTCGAAAGTGCCGGTGGTCGACGGTCGCAAAACCGATGGCAAAAGACTCC
GAGTTCACCCGCTACGTTCCGGTCCCGAAGAACCCCCAGCGGCCTCAACACCTCATCCGACG
GCAAGTACTTCATTGCCAACGGCAAGCTTTCGCCGACGGTCTCAATGATTGCCATCGACCGTC
TGGACGACCTGTTTGCCGACAAATTCAAGGGACCCGCGCGAGGTCATCATCGCCGAGCCAG
AATTGGGCCTGGGCCCGTTGCACACCACGTTCGACGGTCGTGGCAACGCCTACACCACGTTG
TTCATCGACAGTCCAGGTCGTGAAGTGGAACATGGAAGAAGCGATTCGCGCCTACAAGGGG
CGAAAAGGTCAATTACATCAAGCAAGGAAGCTCGACGTGCATTACCAGCCCGGCCATAACCA
TGCCTCGCTGACCGAAACCAGTGAAGCGGACGGCAAGTGGCTGATGGTGTTGTGCAAGTTC
TCCAAGGAC

Translated AA sequence of NosZ

TGWWCSHSAYRGRDHGPASSSTWTARKCRWSTVAKPMAKDSEFTRYVPVPKNPQRPQHLIR
RQVLHCQRQAFADGLNDCHRPSGRPVCRQIQGTRARSSSPSQNWAWARCTPRSTVVATPTPR
CSSTVQVVKWNMEEAIRAYKGRKGQLHQARKLDVHYQPGHNHASLTETSEADGKWLMVLCK
FSKDX

Figure 3.7 Detection of *nosZ* gene-like DNA by PCR using a degenerate nosZ gene-specific primer pair.

PCR assay for nosZ gene on an incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B and a complete denitrifier *Pseudomonas* sp. 10CFM5-2B were performed as described in Materials and methods, using a thermo-gradient PCR set its annealing temperature at 44, 46, 48, 50, 52, and 54 ℃. The partial sequence of 546 bases in the partial *nosZ* amplicon from 10CFM5-2B was determined, and its translated 188 amino acid sequence in NosZ protein, and matched by tblastn to peptide segments on NosZ of denitrifying *Pseudomonas* spp., such as a domain of YQPGHNHASLTETSEADGKWL. Common sequences of the partial NosZ were underlined.

N_2O production was measured in a series of autoclaved media with a range of pH values (3.6-7.6). The pH range of the media decreased to 3.5-6.7 after incubation. All tested strains showed a positive correlation between N_2O production and acidity in the pH range 4.8 to 7.6 (Takeda et al., 2012). Although the amount of N_2O production was low, *Pseudomonas* sp. 10CFM 5-2D showed N_2O emission after incubating in the neutral pH range of 5.4-6.7. N_2O emission was sharply decreased at pH 4.8 and became almost zero below pH 4.5 (Fig.3.8). This trend was similar with that of *Leptothrix* sp. isolated from the spring Pasture soil in 2008 (Takeda et al., 2012), while this was unlike other N_2O emitters much adapting to acidic soils (e.g. *Janthinobacterium* sp. and *Paenibacillus* sp.) (Hashidoko et al., 2008; Takeda et al., 2012). Similarly, trends of N_2O emission potentials of soil microbiota in the post-harvest soils and pure-cultured denitrifiers from the soils were totally opposite to those of soil microbiota in the spring soils.

Among the two N_2O emitters, *Pseudomonas* sp. 10CFM5-1B showed N_2O production in a wide pH range of 4.8–7.6, but at pH 4.6 or below N_2O emission decreased sharply, nearly to zero. The amount of N_2O production changed slightly by increasing of pH from 4.8 to 7.6, but the correlation between N_2O production and pH value (4.8-7.6) was not significant. The optimal pH for N_2O emission from *Pseudomonas* sp. 10CFM5-2D was between 5.4 and 7.6. Emission decreased sharply in the range of pH 4.6 to 3.6 because of low cell growth.

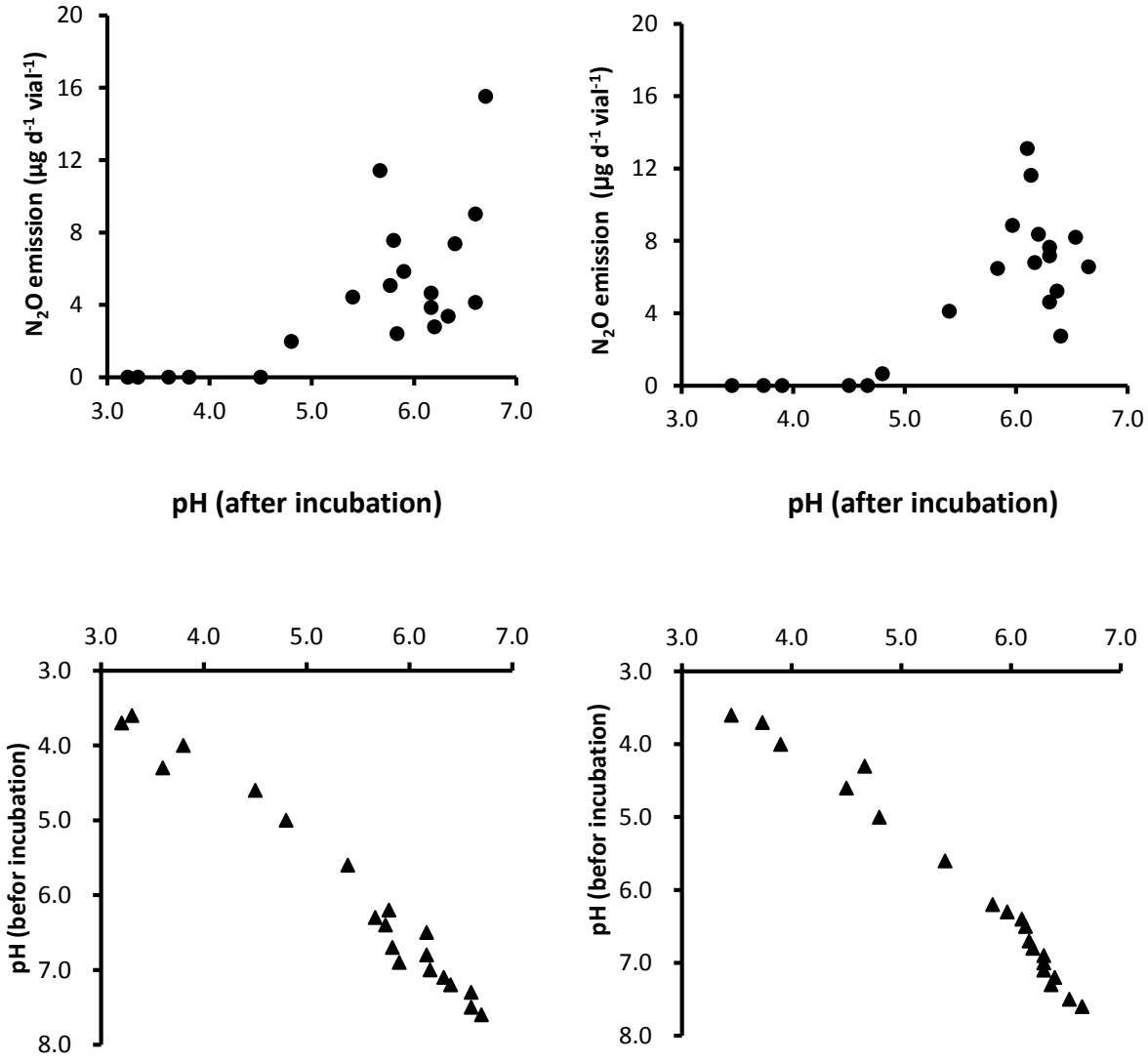


Figure 3.8 Response of two N_2O emitters to various pH of the medium.

Responses of two N_2O emitters *Pseudomonas* sp. 10CFM5-1B (A) and *Pseudomonas* sp. 10CFM5-2D (B) to various pH values were tested in the Winogradsky's gellan gum medium that was supplemented with 36 mM KNO_3 and 1.5 mM sucrose. Each N_2O emitter was pre-cultured on a PDA-KNO_3 plate that was inoculated with 10^6 cells. Cultures of the N_2O emitters were incubated for 7 days in a range of pH values (3.6–7.6) before the incubation. The amounts of emitted N_2O are indicated on the y-axis. Although the cultured medium tended to increase slightly in acidity, particularly at pH 7.0–7.6, the initial pH values were basically maintained after the incubation.

3.2 Effect of C-source and N-source on the diversity of eubacteria associated with N_2O emission

3.2.1 Effect of carbon and nitrogen source on diversity of eubacteria and N_2O emission in soil incubation assay

In the preliminary soil incubation assay carried out in duplicate (Fig. 3.9), supplementation of 1.5 mM sucrose to 5 g soil led to an N_2O emission at 46 ng N_2O $vial^{-1}d^{-1}$ (i.e., approximately 7-fold higher production than the control [6 ng N_2O $vial^{-1}d^{-1}$]) (Fig. 3.9 B). In the absence of sucrose, the N_2O production was only 1.8- and 1.3-fold higher than the control in presence of supplemental NH_4^+ and NO_3^-, respectively, when 0.1 mM (mol per kg of raw soil) inorganic nitrogen was added to 5 g soil as the substrate for N_2O. In addition, NH_4NO_3 showed a combined effect of both NH_4^+ and NO_3^- to be approximately 2-fold of N_2O emission from the control sample.

Conversely, a synergistic effect of the supplemented mineral nitrogen (NH_4^+, NO_3^- or their equivalent mixture at 0.1 mM) and 1.5 mM sucrose was also observed. From the soil in a vial that contained 1.5 mM sucrose and 0.1 mM KNO_3, N_2O gas emitted from the medium (185.8 ng N_2O $vial^{-1}$ d^{-1}) was 29-fold higher than the control (soil only) and 4-fold of the soil supplemented solely with 1.5 Mm sucrose. In presence of 0.1 mM NH_4NO_3 with and without sucrose, N_2O emission was 31- and 4-fold higher N_2O emission, respectively. Under the sucrose and $(NH_4)_2SO_4$, N_2O production from the medium was 22-fold and 3-fold higher than the control and soil only supplemented with sucrose. Despite this drastic change in N_2O emission, all the soils including the control showed almost the same DGGE profile (Fig. 3.9 C).

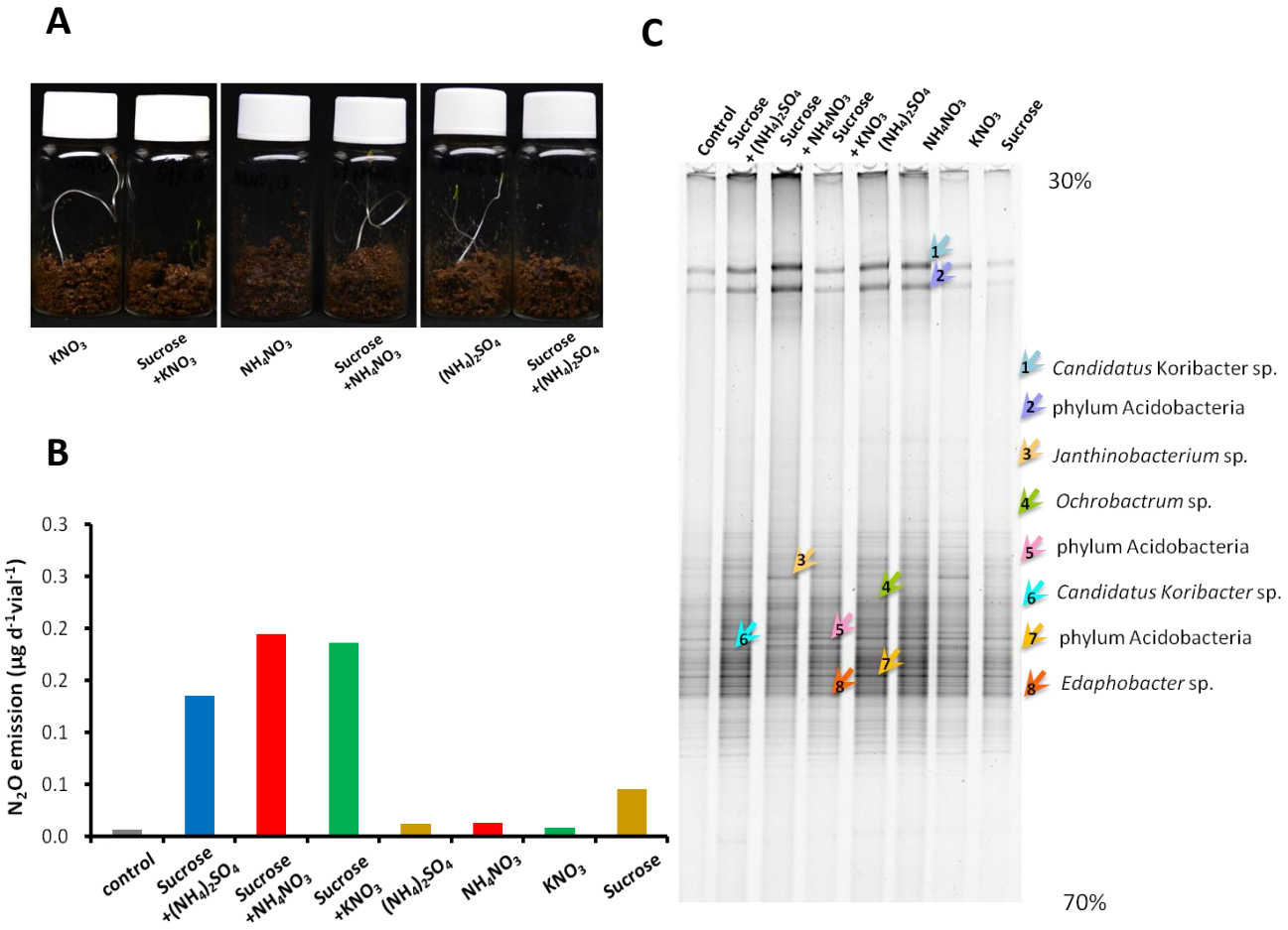


Figure 3.9 Effects of supplemented C- and N-sources on N_2O emission on Andisol from post-harvest corn farm and their microbial community structures.

Soil samples were collected from the Shizunai Experimental Livestock Farm with different fertilizer and manure treatments. (A) A 5 g portion of wet, raw Andisol sample was placed in a 30-ml gas-chromatographic vial. Each treatment was carried out in duplicate. (B) After 4-day-incubation, a 1 ml volume of headspace gas (27.5 ml) was analyzed by ECD-gas-chromatography. The column shows the average of two measurements. (C) DNA was extracted from one of the two soil samples from each treatment, and subjected to 16S rRNA gene-targeted DGGE analysis to compare their bacterial community structure. Some major DGGE bands were sequenced and their homologs were identified by searching against a DNA database. DNA bands successfully identified by genus level are listed alphabetically in the right. Eubacteria identified in the soils supplemented with sucrose and the substrates are three bacteria of phylum Acidobacteria, two *Candidatus* Koribacter sp., *Ochrobactrum* sp., *Janthinobacterium* sp., and *Edophobacter* sp.

3.2.2 Eubacteria DGGE profiles of the culture inoculated with soil suspension

N_2O emission of soil suspension in cultured medium with KNO_3 and sucrose was analyzed. As shown in Fig. 3.10 B, both in season autumn 2011 and spring 2012, the soil samples 10CFM exhibited higher N_2O emission than CK. 10CFM at 15 cm showed the N_2O emission in spring up to 13 μg $d^{-1}vial^{-1}$, which is 4-fold higher than CK 15 cm. Similarly, N_2O emission of 10CFM at 15 cm in spring also exhibited 6-fold higher than in autumn. N_2O emission from 10CFM at 5 cm is 20-fold and 6-fold higher than CK at 5 cm in autumn and spring separately.

The DGGE patterns of eubacteria in the cultured medium inoculated with soil suspension under different fertilization are presented in Fig. 3.10. A distinguishable DNA bands were observed in which existed the high diversity of bacteria, dependent on the presence of nitrogen fertilizer and manure. Dominant DGGE bands detected in the fertilized soil were sequenced. Phylogenetic analysis revealed that, the main denitrifying bacteria were *Burkholderia* sp. and *Massilis* sp. in presence of fertilizer and manure, while *Paenibacillus* sp. was only detected in soil 10CFM at 15 cm (Fig. 3.10C). *Janthinobacterium* sp. inhabited in all soil samples, but only in CK at 5 cm soil (without fertilization), this bacterium is the dominant species.

Seasonal differences also had a significant influence on the diversity for the denitrifiers responded to active N_2O production, as shown in Fig. 3.10C, and the high diversity and abundance of denitrifier *Burkholderia* sp. inhabited in 10CFM at 15 cm in April, leading to active N_2O production. However, compared to the soil in spring 2012, the appearance of *Janthinobacterium* sp. in 10CFM at 5 cm and 10CFM at 15 cm in autumn 2011, did not show any acceleration effect on N_2O emission. The similar trend was observed in CK treatment, in which the dominant band was identified as *Burkholderia* sp. in CK at 15 cm in spring 2012 which exhibited high N_2O emission. Conversely *Massilis* sp. was also the main bacterium in CK at 15 cm in autumn, but it showed the low N_2O production.

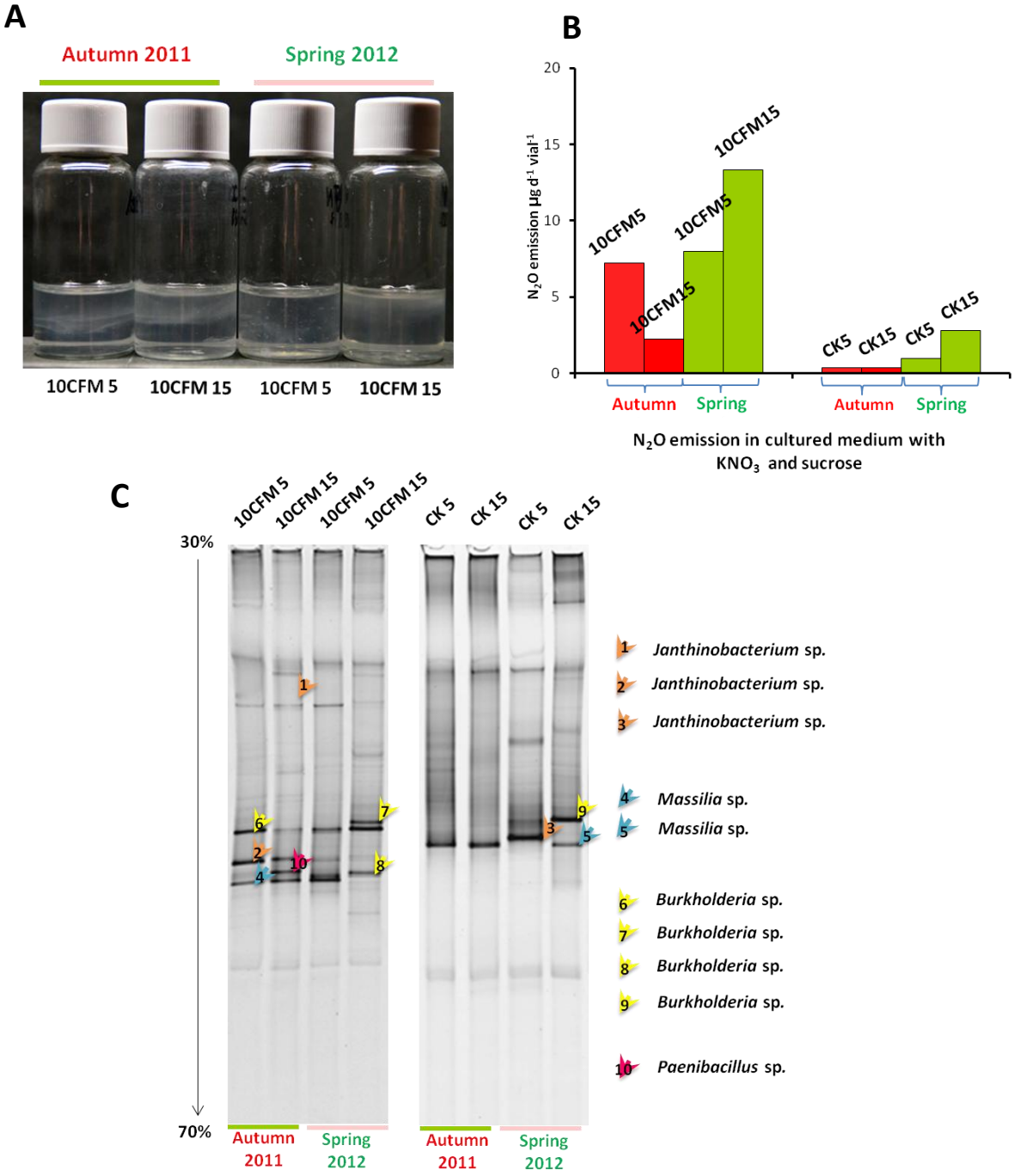


Figure 3.10 N_2O emission and DGGE profiles of the culture inoculated with soil suspension.

Soil samples 10CFM and CK were collected from the Shizunai Experimental Livestock Farm in autumn 2011 and spring 2012. 10CFM 5 and 10CFM15: soil applied with fertilizer and manure from 2010 at 5 cm and 15 cm; CK5 and CK15: soil without any fertilization at 5 cm and 15 cm

(A) Soil suspension incubated in medium supplemented with 0.05% sucrose and 50 mg L^{-1} NO_3^--N incubated at 20℃ for 7 days in dark. (B) N_2O emission of incubated soil suspension. (C) DGGE profiles of denitrifier community structures. Different color of arrows indicated different species of denitrifiers. *Burkholderia* sp. and *Massilia* sp. are the main species in the cultures.

3.2.3 DGGE profiles and N_2O emission of soil suspension cultured in optional C- and N- sources

The DGGE profiles and N_2O emissions of soil suspension cultured in medium with optional carbon and nitrogen sources were analyzed. From the Fig. 3.11 A, addition of sucrose and KNO_3 significant accelerated N_2O emission up to 2.2–13.3 μg $d^{-1}vial^{-1}$, in contrast, N_2O emissions from control (without sucrose and KNO_3) and only supplemented with sucrose are no more than 0.1 μg $d^{-1}vial^{-1}$. However, addition of sucrose showed relatively higher N_2O emission than control and the culture supplemented with sucrose only. However, the N_2O production was still much lower than that under the presence of sucrose and KNO_3. For seasonal difference of N_2O production potential, control and the culture with addition of sucrose did not show any differences between autumn soil and spring soil because of low N_2O emission, while in presence of both sucrose and KNO_3, more active N_2O emission was observed in soil collected in spring 2012 rather than autumn soil 2011 (Fig. 3.11 A).

To investigate the differences of N_2O emission under carbon and nitrogen sources, the diversity of soil denitrifiers were furthermore analyzed. From the DGGE profiles, in the presence of both sucrose and KNO_3, a clear shift of dominant communities was observed. In control and culture supplemented with sucrose, the dominant bands remained unchanged and were identified as *Janthinobacterium* sp. that exhibited no active N_2O emission, whereas addition of sucrose increased the intensity of *Duganella* sp. (Fig. 3.11 B). Addition of KNO_3 led to increase abundance and intensity of *Burkholderia* sp. and *Variovorax* sp., whereas decreased the abundance of *Janthinobacterium*. A clear change on diversity of the denitrifiers was observed in the presence of sucrose and KNO_3, and the main candidate denitrifers were *Burkholderia*, *Massillia* and *Janthinobacterium*. An increased diversity and relatively high intensity of *Burkholderia* sp. which attributed to high N_2O emission was observed in 10CFM both in autumn 2011 and spring 2012, while *Janthinobacterium* sp. showed a drastic disappearance. In addition, some special bands, such as *Paenibacillus* sp., were also detected in the presence of sucrose and KNO_3 (Fig. 3.11 B).

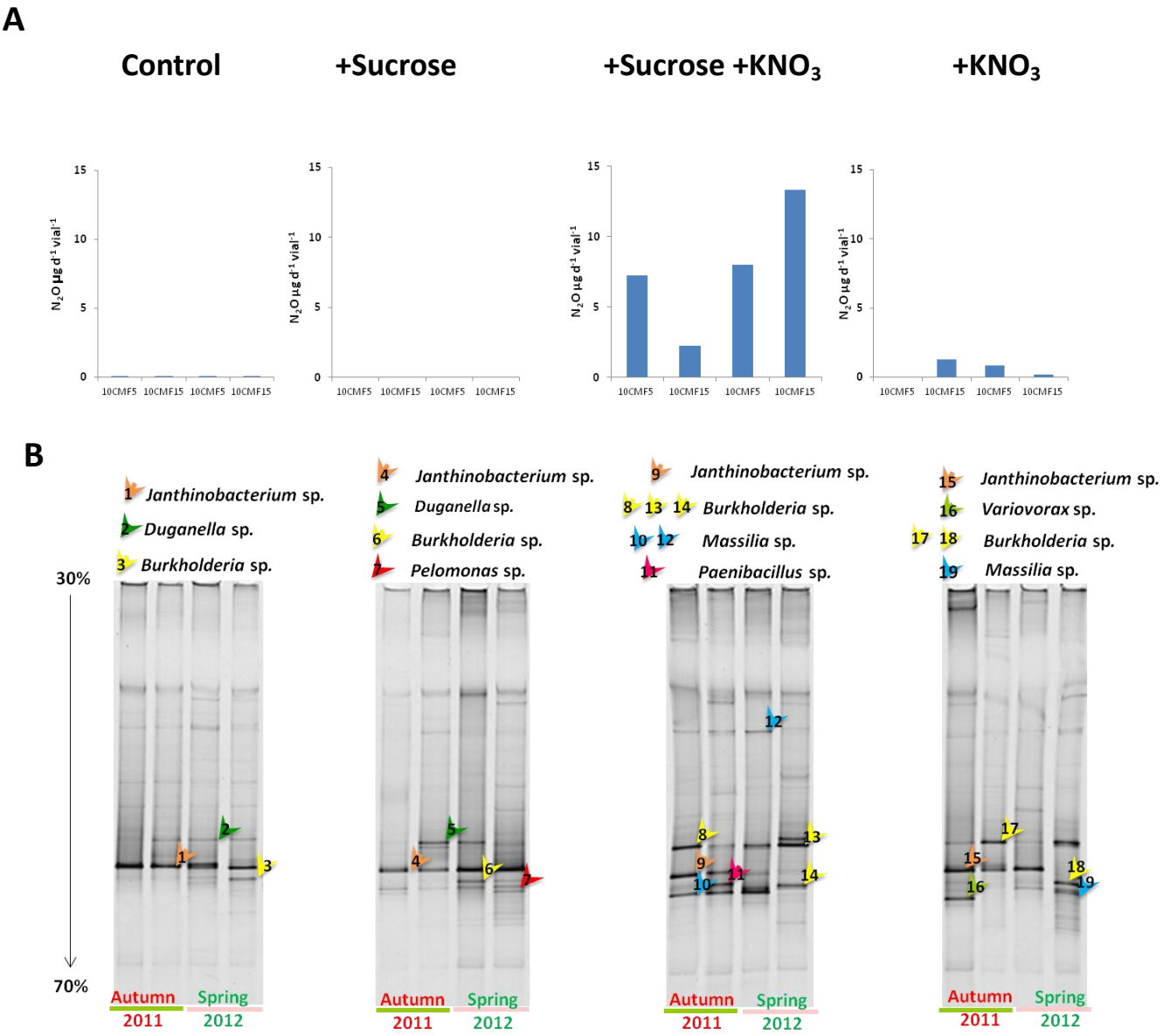


Figure 3.11 N_2O emission and DGGE profiles of soil suspension cultured in optional C- and N- sources.
Soil samples 10CFM 5 and 10CFM 15 were collected from the Shizunai Experimental Livestock Farm in autumn 2011 and spring 2012. (A) N_2O production of soil suspension cultured in alternative medium of carbon and nitrogen sources. Control: Winogradsky's medium without any carbon and nitrogen sources; +sucrose: medium supplemented with 0.05% sucrose; +sucrose +KNO_3: medium supplemented with 0.05% sucrose and 50 mg/L (0.36g l^{-1} KNO_3) NO_3^--N; +KNO_3: medium supplemented with 50 mg/L NO_3^--N. (B) DNA was extracted from medium from each treatment, and subjected to 16S rRNA gene-targeted DGGE analysis to compare their bacterial community structures.

3.2.4 Bacterial succession of soil culture in alternative N-sources by PCR-DGGE

In order to investigate the N_2O emission and bacterial succession of soil culture in alternative nitrogen sources, 10 mg of Andisol was inoculated to 10 ml medium supplemented with different nitrogen-sources and incubated at 20℃ for 7 days (Fig. 3.12 A). In the presence of 0.05% sucrose, when 5 mM (mole kg^{-1} of raw soil) inorganic nitrogen was added as the substrate for N_2O to the medium inoculated with 10 mg soil, the N_2O production was significantly higher than the control (only supplemented with 0.05% sucrose). Addition of KNO_3 exhibited the highest N_2O production among all the cultures up to 12.5 μg $d^{-1}vial^{-1}$, which is 13- and 10- folds higher than those supplemented with NH_4NO_3 and $(NH_4)_2SO_4$, respectively (Fig. 3.12 B).

Analysis of DGGE profiles of diverse soil nitrogen fixers and denitrifiers associated with N_2O production showed a clear shift under different nitrogen sources. In the absence of ammonia and nitrogen sources, *Clostridum* spp. were detected as main nitrogen fixers. In addition, oligotrophic bacterium *Massilia niastensis* was also detected (Fig. 3.12 C). However, the presence of all these three substrates, KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$, saprophytic β-proteobacteria *Burkholderia* became newly dominant bacteria, leading to active N_2O emission, whereas some species, such as two *Clostridium* and a *Massilia* sp. disappeared in the gel culture. Furthermore, the addition of ammonia did not affect population density of *Burkholderia* sp., while allowed emergence of an ammonium oxidizer *Arthrobacter* which can oxidize ammonium to hydroxylamine, probably leading to low N_2O production (Fig. 3.12). In contrast, the highest N_2O emission was observed in the presence of 0.05% sucrose and 5 mM KNO_3 because of the relatively high diversity and abundance of denitrifiers *Burkholderia* sp. associated with N_2O emission (Fig. 3.12 C). From this result, it is also shown that *Burkholderia* sp. plays an important role in N_2O production.

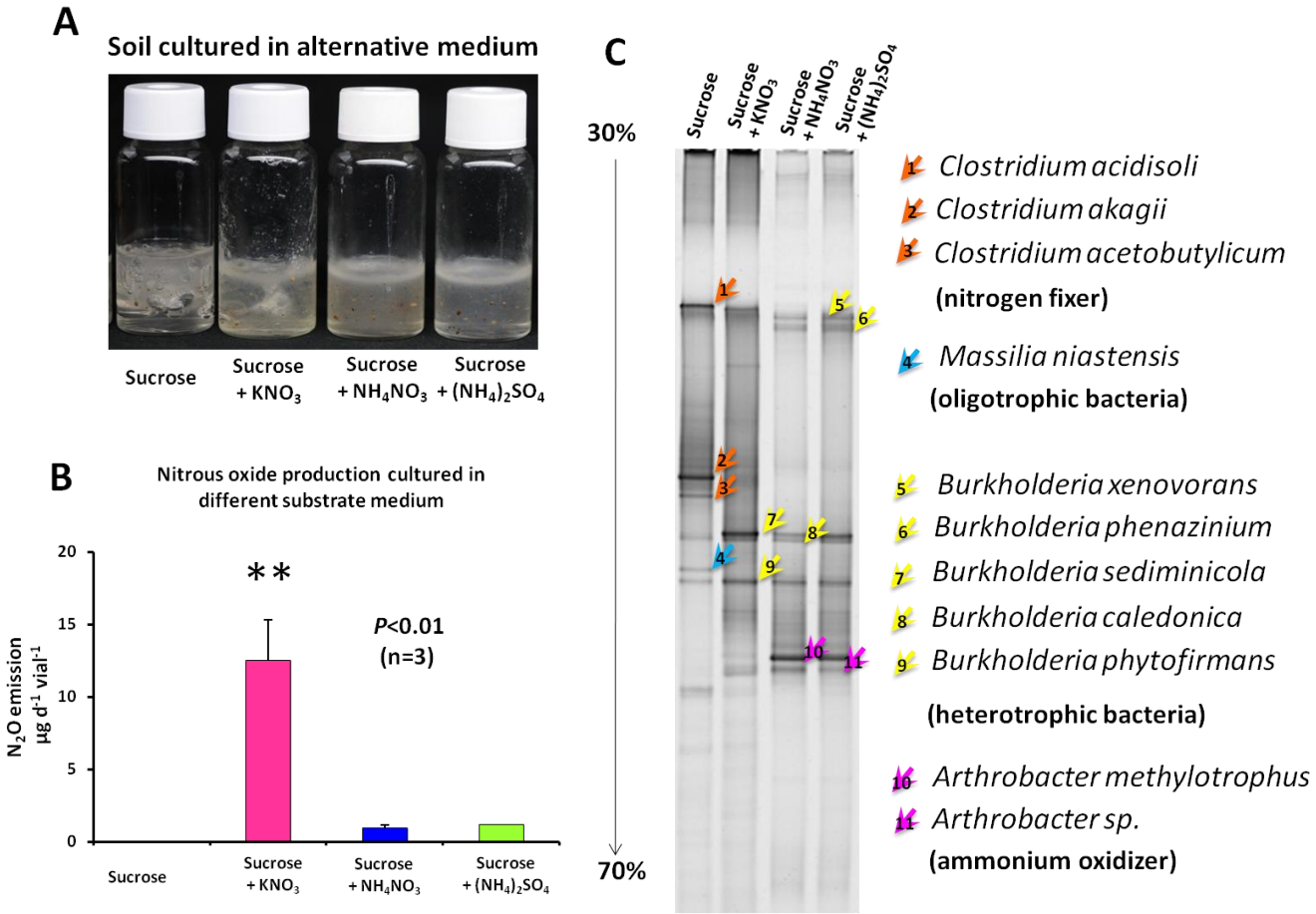


Figure 3.12 Bacterial succession of soil cultured in alternative N-sources by PCR-DGGE.

Soil samples 10CFM5 collected from the Shizunai Experimental Livestock Farm in autumn 2011 was used as the test soil. (A) 10 mg fresh Andisol from treatment 10CFM was suspended into 10 ml medium, vortexed for 1 min until it become homogeneous, and then incubated at 20 ℃ for 5 days in the dark. Winogradsky's mineral medium supplemented with alternative substrate of N_2O are shown as follows: KNO_3 (5 mM), NH_4NO_3 (5 mM), $(NH_4)_2SO_4$ (2.5 mM). A 0.05% sucrose was used as carbon source. Each treatment was in triplicate. (B) N_2O production from the medium under each treatment. Values are means ± SD (shown by error bars) (n = 3). $^{**}P < 0.01$ by Student's *t*-test. (C) DNA was extracted from the soil culture in each medium, and subjected to 16S rRNA gene-targeted DGGE analysis.

3.2.5 Effect of C-source on the diversity of eubacteria and N_2O emission

It is known that carbon (C) amendments increase microbial activity and induced changes in abundance of total bacterial communities particularly those of denitrifiers in studies of anoxic soil microcosms. The diversity of bacterial communities associated to N_2O emission in the absence and presence of carbon source and/or alternative nitrogen sources were investigated.

In presence of nitrogen source of NO_3^-, addition of 0.05% sucrose obviously accelerated N_2O emission up to 12.5 μg $d^{-1}vial^{-1}$, more than 2-fold higher than that without sucrose (Fig. 3.13A). The diversity and abundance of denitrifer community, including *Burhoderia* sp., was increased, leading to higher N_2O emission in the sucrose and NO_3^- amendment medium, whereas oligotrophic bacterium *Rhodanobacter* sp. emerged without supplementation of sucrose (Fig. 3.13B). The similar trends were also observed under the addition of NH_4NO_3 or $(NH_4)_2SO_4$. Addition of 0.05% sucrose increased abundance and diversity of dominant denitrifiers *Burkholderia* spp. and ammonium oxidizer *Arthrobacter* spp., while sucrose supplementation resulted in suppression of oligotrophic bacteria *Rhodanobacter* sp. (Fig. 3.13C).

In contrast, the addition of sucrose did not show any accelerating effect on N_2O emission in presence of NH_4NO_3 and $(NH_4)_2SO_4$ as shown in Fig. 3.13A, N_2O emission of soil culture in the sucrose and 5 mM NH_4NO_3 is only 0.9 μg $d^{-1}vial^{-1}$, while it showed up to 10.1 μg $d^{-1}vial^{-1}$ in the absence of sucrose. The emergence of ammonium oxidizer *Arthrobacter* sp. under the supplementation with ammonia and sucrose suppressed N_2O production probably because the presence of carbon source with ammonium resulted in nutritional competition with N_2O emitters (Fig. 3.13C). In addition, the plate culture of the incubated medium indicated that under the presence of sucrose and ammonia (Fig. 3.13B), fungi can dominantly grow (compare to nitrate-supplemented culture), which suppressed the growth of bacteria, leading to lower N_2O production than others.

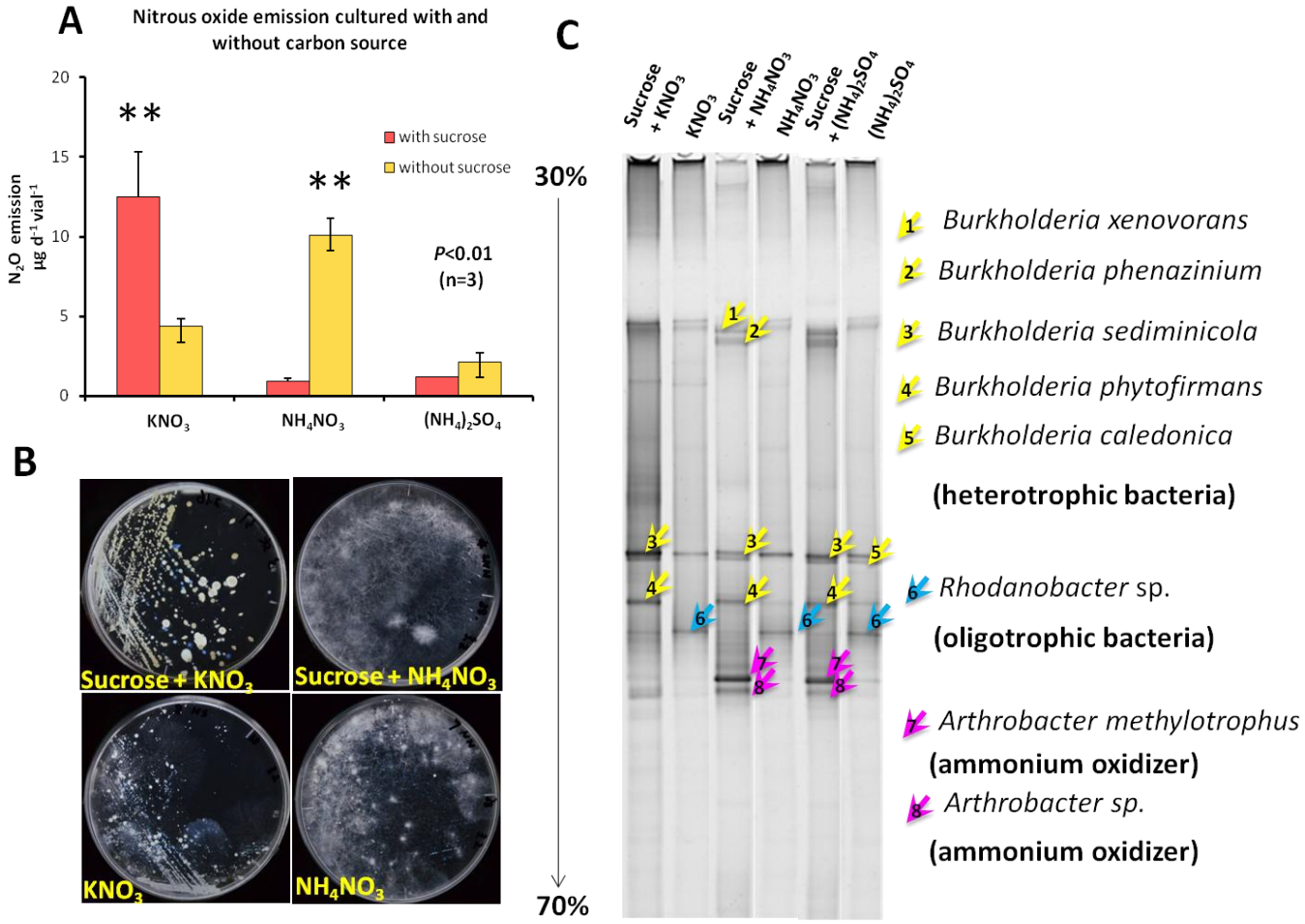


Figure 3.13 Effect of C-source on the diversity of eubacteria and N_2O emission under alternative N-source.

Soil samples 10CFM 5 collected from the Shizunai Experimental Livestock Farmland in autumn, 2011 was used as the test soil. (A) N_2O production for each treatment. A 10 mg fresh Andisol from 10CFM was added to 10 ml medium supplemented with alternative N-sources and sucrose, and then incubated at 20 ℃ for 5 days in the dark. The concentrations of N-source are determined as follows: KNO_3 (5 mM), NH_4NO_3 (5 mM), $(NH_4)_2SO_4$ (2.5 mM). As a carbon source, 0.05% sucrose was used. Values are means ± SD (shown by error bars) (n = 3). $^{**}P < 0.01$ by Student's *t*-test. (B) Growth performance of soil microorganisms on MWG plate. A portion of 10 μl cultured medium was spread on MWG plate, and incubated at 20 ℃ for 7 days. Fungus grew well in the presence of NH_4^+, especially under the supplementation of sucrose. (C) DNA was extracted from cultured medium of each treatment, and subjected to 16S rRNA gene-targeted DGGE analysis. *Burkholderia* sp. and *Arthrobacter* sp. are the dominant species in presence of sucrose in the culturing medium.

3.3 Effects of chemical compounds on bacterial N_2O emission

3.3.1 Inhibitory effects of methyl viologen dichloride (Paraquat®) and other chemical compounds on N_2O emission

As it has been reported that methyl viologen radicals are powerful inhibitors against CH_4 production by methanogens, such as *Methanobacillus omelianskii* (Wolin et al., 1964), the author first expected that methyl viologen dichloride would act as an inhibitor of nitrous oxide reductase to accelerate N_2O production; therefore, the author attempted to employ this herbicide for a positive control. Contradictory to the hypothesis, methyl viologen dichloride showed a high inhibitory activity against N_2O emission by a tested denitrifier, with almost no emission at 10 μM (Fig. 3.14).

Some reports suggested that corn farm soils efflux N_2O more actively than other crops (Francesco Alluvione et al., 2009). As it is also known that, corn produces N-heterocyclic secondary metabolites, such as 6-methoxy-2-benzoxazolinone (MBOA). Therefore, the impact of other herbicides and chemicals related to corn antifungal metabolites on the denitrification process with pseudomonad denitrifiers was further investigated. MBOA, an allelochemical of corn, slightly repressed N_2O emission of *Pseudomonas* sp. 10CMF5-1B at 10 μM. In contrast, 1-hydroxy-1*H*-benzotriazole (HOBt), a couping reagent for amide synthesis used for a negative control, did not show any statistically significant inhibitory effects on N_2O emission at 10 μM and 100 μM by N_2O emitters *Pseudomonas* sp. as expected (Fig. 3.14). The similarly results were also observed in 2-benzoxazolinone (BOA), an oxidation-stress inducer. BOA at 10 μM and 100 μM exhibited lower N_2O production by *Pseudomonas* sp. 10CFM5-1B, but the repression was not statistically significant (Fig. 3.14).

Both the BOA and HOBt performed as negative controls without any significant repression or acceleration of N_2O production. Therefore, more accurate dose responses of methyl viologen, MBOA, HOBt toward the N_2O-emitting bacteria were examined.

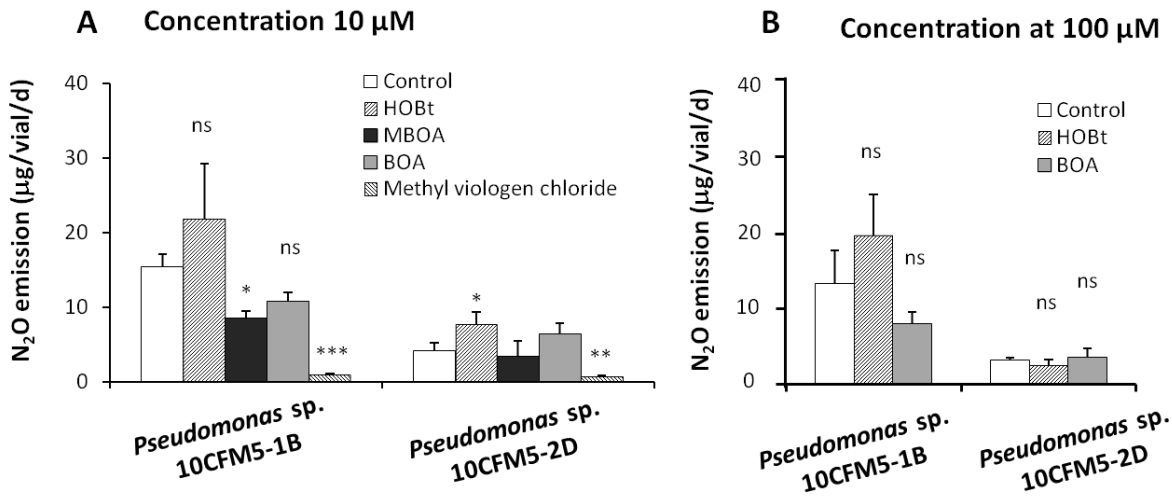


Figure 3.14 N_2O production by N_2O-emittable *Pseudomonas* sp. upon exposure to the chemical compounds methyl viologen dichloride, MBOA, BOA, and HOBt.

The N_2O emitter *Pseudomonas* sp. 10CFM5-1B was used in the presence of 10 µM (A) or 100 µM (B) methyl viologen dichloride, MBOA, BOA, or HOBt. The culture medium was supplemented with 0.5 g L^{-1} NO_3^- form of N (36 mg KNO_3 in 10 mL medium) and 0.05% sucrose (5 mg in 10 mL). Culture conditions were at 25 ℃ in the dark for 7 days. The values are means ± SD (shown by error bars) (n = 3). Methyl viologen dichloride showed statistically significant suppression of N_2O production at 10 µM (**P* < 0.05, ****P* < 0.001, by Student's *t*-test); therefore, inhibition tests at higher concentrations were not performed.

3.3.2 Dose-dependent effect of methyl viologen chloride on N_2O emission

According to the preliminary results, methyl viologen dichloride significantly inhibited N_2O emission at 10 μM concentration, then we examined more accurate dose-dependent assay of methyl viologen chloride by N_2O emitters *Pseudomonas* sp. 10CFM5-1B and *Pseudomonas* sp. 10CFM5-2D. Dose-dependent effect of methyl viologen dichloride on N_2O emitters was conducted at concentration among 1-5 μM, leading to a complete inhibition of N_2O emission. Even at 1 μM concentration, methyl viologen showed 50% suppression of N_2O emission compared to the control, reduced from 26.8 to 10.5 μg d^{-1} $vial^{-1}$ for *Pseudomonas* sp. 10CFM5-1B and 10.9 to 3.9 μg d^{-1} $vial^{-1}$ for *Pseudomonas* sp. 10CFM5-2D, respectively (Fig. 3.15). With the increase concentration of methyl viologen dichloride from 0.5-2.0 μM, the N_2O emission decreased drastically.

The bacterial cell growth performance of *Pseudomonas* sp. 10CFM5-1B in the presence of methyl viologen dichloride was shown in soft gel medium in the vials (Fig. 3.15). Compare with the control, cell-growth inhibition of the denitrifier was rarely observed the test bacteria exposed to methyl viologen dichloride in the range from 2-5 μM.

3.3.3 Dose responses of the pseudomonad N_2O emitters toward HOBt and MBOA

A more accurate test for the inhibition of N_2O emission was performed for two benzo-*N*-heterocyclic compounds (HOBt and MBOA) in triplicate. MBOA showed a statistically significant repression of N_2O emission at 10 and 50 μM (Fig. 3.16). In addition, HOBt at 150–500 μM reduced N_2O production to almost null, but the culture medium containing 150 μM or higher concentration of HOBt inhibited the cell growth of *Pseudomonas* sp. 10CMF5-1B (Fig. 3.16). Repression of N_2O production by exceptionally high concentration of the chemical with the inhibition of bacterial cell growth is not pin-point repression or inhibition of N_2O emission. Therefore, such N_2O

emission is meaningless as N_2O production inhibition. BOA also showed toxicity similar to that of HOBt (data not shown).

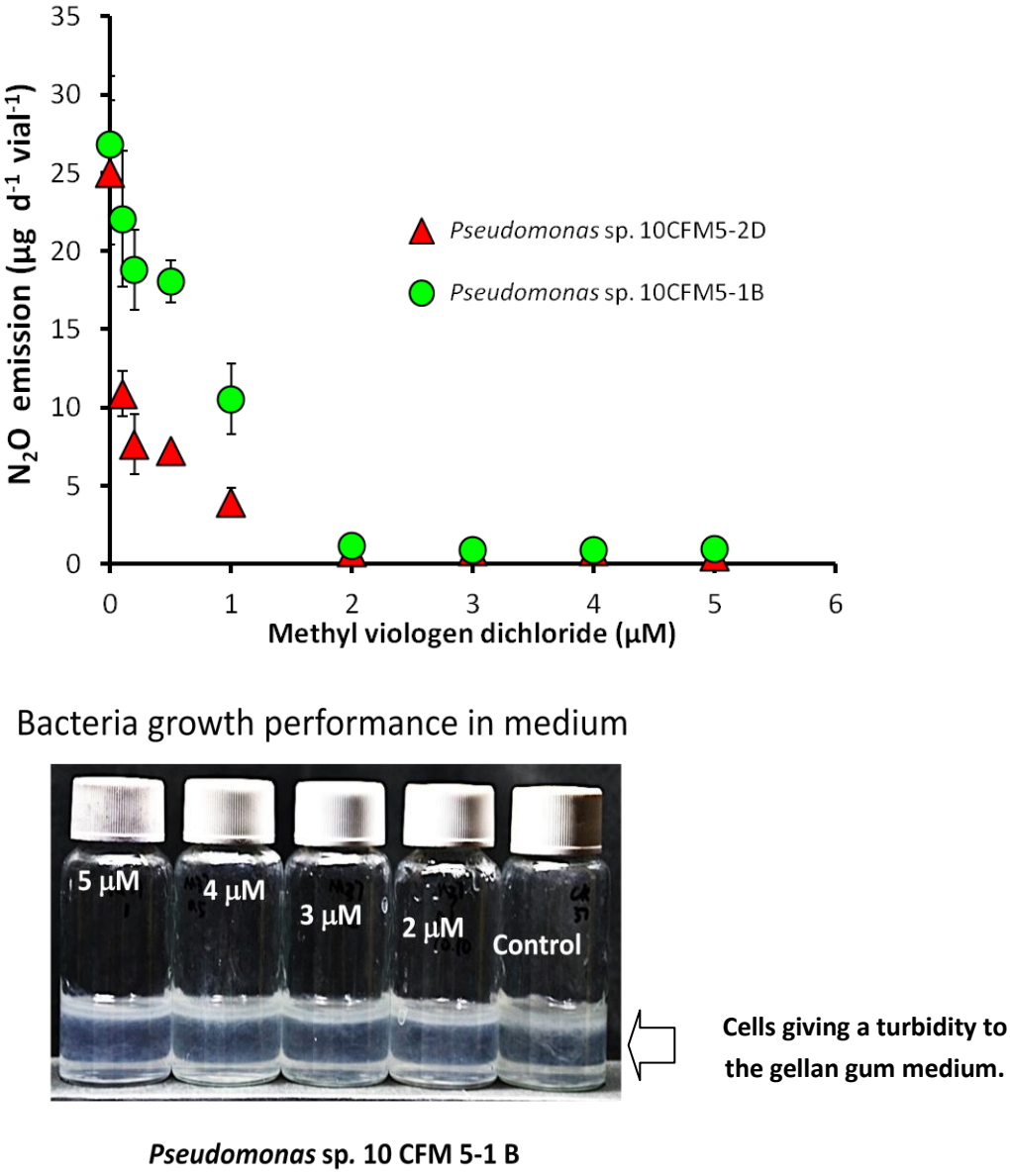


Figure 3.15 Dose response of methyl viologen dichloride towards N_2O production by N_2O-emittable *Pseudomonas* sp.

Two N_2O emitters, *Pseudomonas* spp. 10CFM5-1B and 10CFM5-2D, were used. The culture medium was supplemented with 0.5 g L^{-1} NO_3^- form of N (36 mg KNO_3 in 10 mL medium) and 0.05% sucrose (5 mg in 10 mL). The culture conditions were at 25 ℃ in the dark for 7 days. Values are means ± SD (shown by error bars) (n = 3).

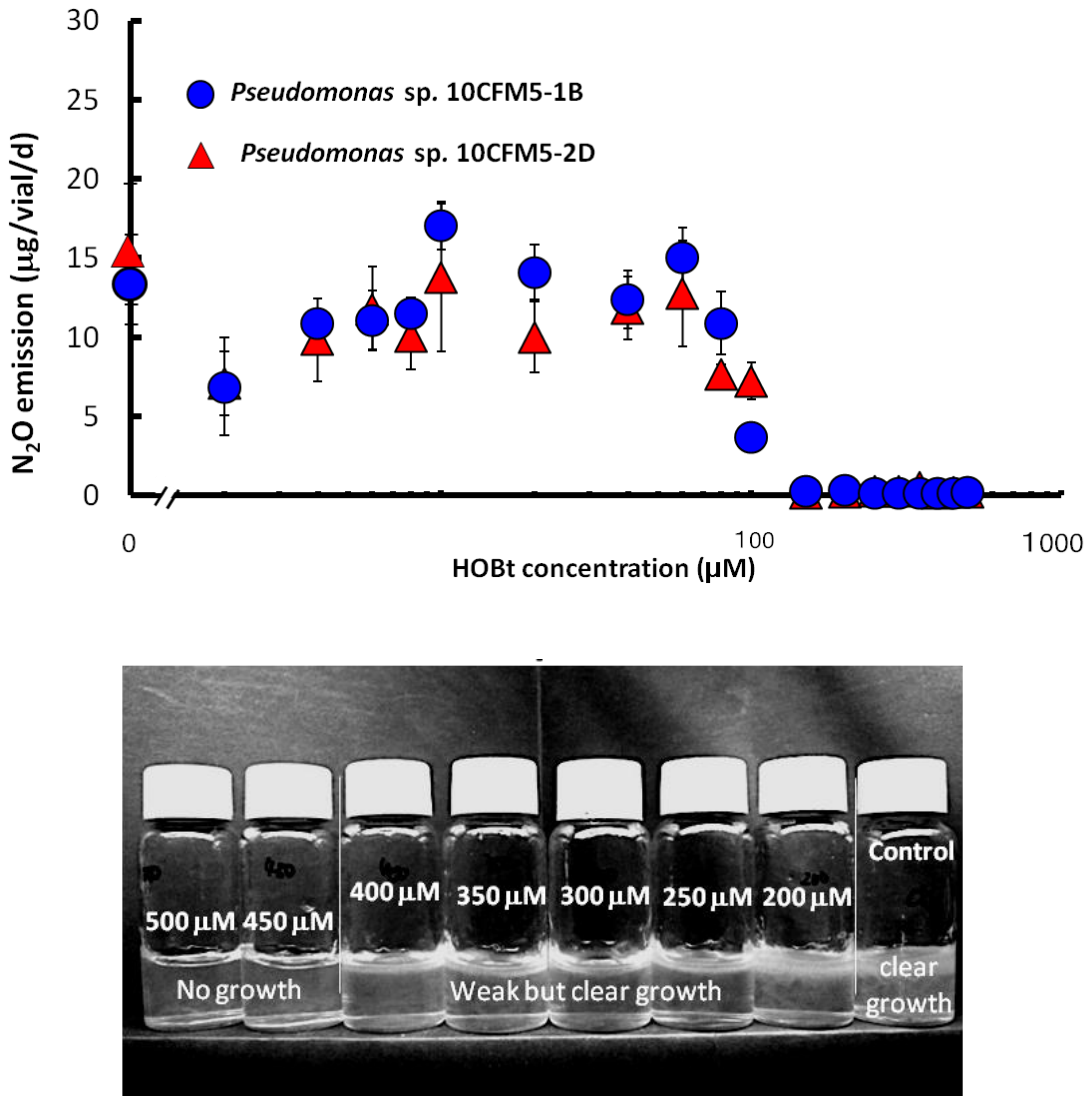


Figure 3.16 Dose responses of the pseudomonad N_2O emitters toward HOBt.

Repression of N_2O production by two *Pseudomonas* N_2O emitters exposure to HOBt was examined in the range from 2.0 to 500 μM. N_2O production was measured in 10 ml soft gel medium supplemented with 36 mg KNO_3 and 5 mg sucrose. Culture conditions were of 25 ℃ in the dark for 7 days. Values are means ± SD (shown by error bars) (n = 3).

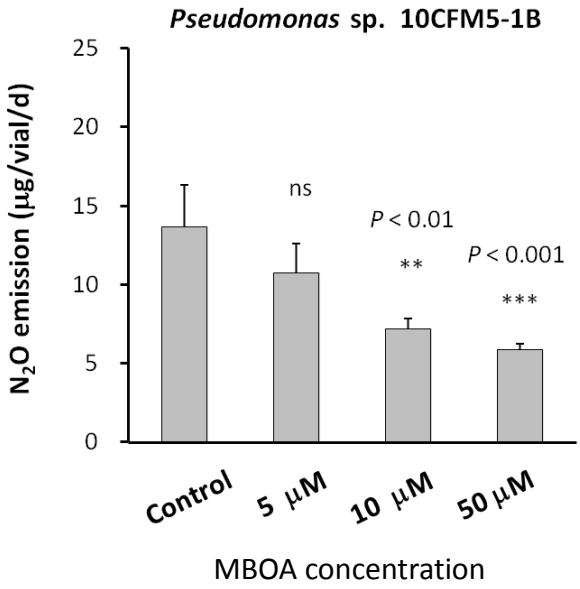


Figure 3.17 N_2O production by N_2O-emittable *Pseudomonas* sp. upon exposure to MBOA.

N_2O emitter *Pseudomonas* sp. 10CFM5-1B was exposed to MBOA at 5, 10, or 50 μM. The culture medium was supplemented with 0.5 g L^{-1} NO_3^- form of N (36 mg KNO_3 in 10 mL medium) and 0.05% sucrose (5 mg in 10 mL). Culture conditions were at 25 ℃ in the dark for 7 days. Values are means ± SD (shown by error bars) (n = 3). Methyl viologen dichloride showed statistically significant suppression of N_2O production at 10 μM (***P* < 0.01, ****P* < 0.001 by Student's *t*-test).

3.3.4 Accelerating effects of amitrol and other chemical compounds on bacterial N_2O emission

Amitrole (3-amino-1*H*-1,2,4-triazol) at 2 μM showed significant acceleration of N_2O production. This acceleration effect, 2.5 fold-higher than that of the control, was observed only in the incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B (Fig. 18). Importantly, the incomplete denitrifying N_2O emitter showed a clear response to amitrol at a low concentration (2 μM). At 10 μM, amitrole rather suppressed N_2O emission of the incomplete denitrifier.

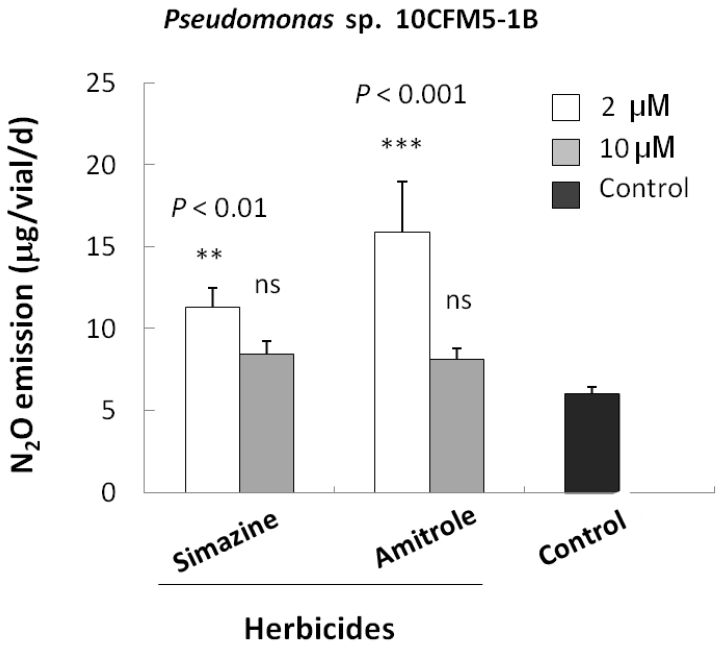


Figure 3.18 N_2O production by *Pseudomonas* sp. 10CFM5-1B in the presence of the herbicides simazine and amitrole.

N_2O production by *Pseudomonas* sp. 10CFM5-1B in the presence of the herbicides simazine and amitrol was tested, along with that of a control. Culture conditions were of 25 ℃ in the dark for 7 days. Values are means ± SD (shown by error bars) (n = 3). ***P* < 0.01, ****P* < 0.001 by Student's *t*-test.

3.4 Effects of root exudates on N_2O emission by pseudomonad denitrifiers

3.4.1 Acceleration effect of corn root extracts on N_2O emission by *Pseudomonas* sp. 10CFM 5-2B

In order to explore the roots that attributed to active N_2O productions, the complete denitrifier *Pseudomonas* sp. 10CFM5-2B and incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B were used as the test bacteria. It is hypothesized that the corn root exudates have an ability directly or indirectly to activate N_2O-emitting soil microorganisms.

Dent corn (Maize) root extracts were added to Winogradsky's medium as supplements and the final concentration of the extract was adjusted to 10 mg L^{-1}, and then inoculated with N_2O emitting bacteria *Pseudomonas* spp. As shown in Fig. 3.19B, addition of the roots extracts significantly accelerated the N_2O emission of complete denitrifier *Pseudomonas* sp. 10CFM5-2B, whereas incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B did not show any acceleration/suppression by the addition of corn root extracts. This stimulation effect on the N_2O emission by addition of root extracts reached to 1.5 fold higher than control by complete denitrifier *Pseudomonas* sp. 10CFM5-2B (Fig. 3.19B).

The growth performance of *Pseudomonas* spp. 10CFM5-1B and 10CFM5-2B in Winogradsky's medium with and without corn extracts was also monitored (Fig. 3.19A). Compared to control (without corn extracts), both of these two N_2O emitters exhibited similar growth performance in the medium supplemented with root extracts, suggested that 10 mg L^{-1} of the root extracts had no acceleration/inhibition effect on bacterial growth.

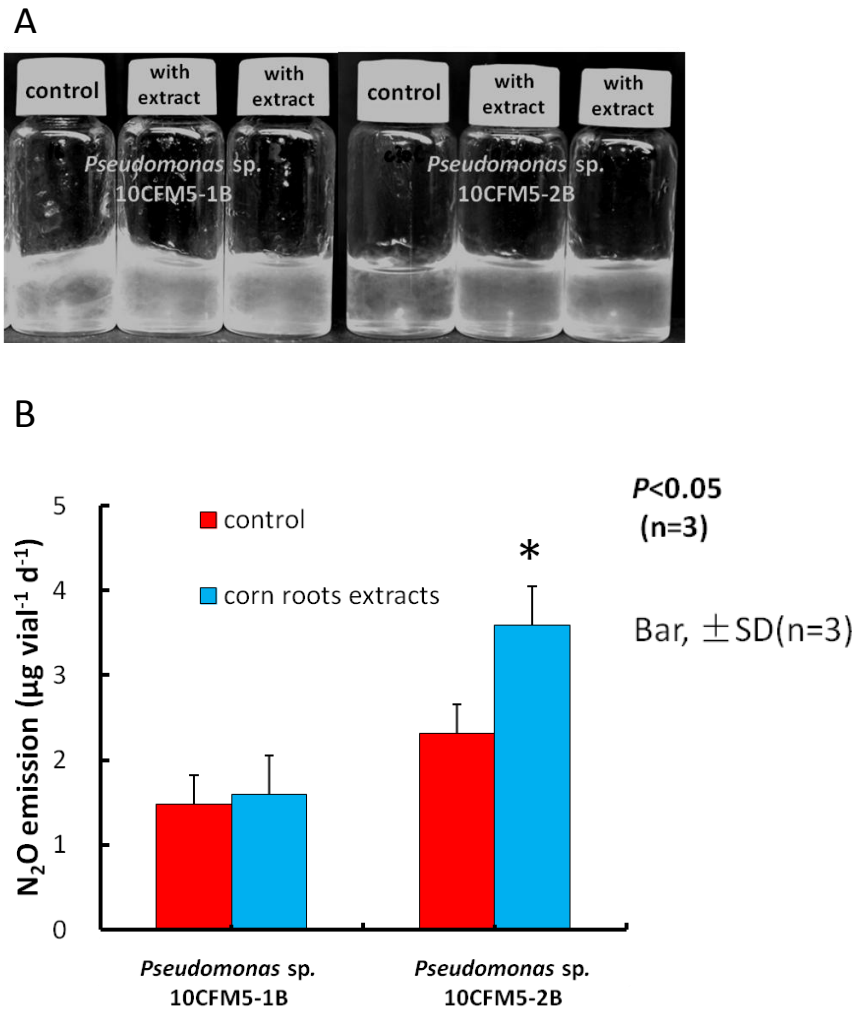


Figure 3.19 Effect of dent corn root extracts on N_2O production by *Pseudomonas* sp. denitrifiers.

The complete denitrifier *Pseudomonas* sp. 10CFM5-2B and incomplete denitrifier *Pseudomonas* sp. 10CFM5-1B were used as the test bacteria. The culture medium was supplemented with 0.5 g L^{-1} NO_3^- form of N (36 mg KNO_3 in 10 mL medium) and 0.05% sucrose (5 mg in 10 mL). (A) Growth performance of denitrifying *Pseudomonas* sp. in the medium supplemented with 10 mg L^{-1} corn root extracts. (B) N_2O production by denitrifying *Pseudomonas* sp. in the presence of corn root extracts. Culture conditions were at 25 ℃ in the dark for 4 days. Values are means ± SD (shown by error bars) (n = 3). **P* < 0.05 by Student's *t*-test.

3.4.2 Effect of root exudates from plant seedling on N_2O emission by *Pseudomonas* sp. 10CFM5-2B

Dent corn is used for animal feed, for starch source to make corn syrup, and for biofuel and bioplastics, while sweet corn known as a vegetable, rather than a grain crop. Dent corn is higher in starch and lower in sugar than sweet corn. Therefore, effect of root exudates from dent corn and sweet corn on N_2O emission by complete denitrifier *Pseudomonas* sp. 10CFM5-2B was investigated, using a soilless culture system mimicking soil conditions, in which 0.3% NH_4NO_3 and 0.3% gellan gum were contained. When NH_4NO_3 was supplied as a nitrogen source and the substrate for N_2O, both ammonium nitrogen and nitrate nitrogen were provide to the test plants.

As expected, in the presence of both dent corn and sweet corn, N_2O emission in the vial drastically increased, up to 0.6 μg d^{-1} $vial^{-1}$ and 0.3 μg d^{-1} $vial^{-1}$ respectively, whereas the N_2O emission in the medium without germinated seedlings is no more than 0.1 μg d^{-1} $vial^{-1}$ (Fig. 3.20B). The bacterial cell growth performance of *Pseudomonas* sp. 10CFM5-2B was shown in transparent soft gell medium (Fig. 3.20A). Consequently, a high population density of the bacteria cells was observed near the gel top surface, exhibiting characteristics as aerobic oligotrophy, while other bacteria gathered around the corn roots, especially in dent corn seedlings. In contrast, the population of bacteria cells was much lower in control (without any seedlings).

Meanwhile, the similar experiments were conducted on hairy vetch (*Vicia villosa*), sorghum (*Sorghum bicolor*) and hey oats (*Avena sativa*). Hairy vetch (*Vicia villosa*) which is used as leguminous cover crops and a green manure is generally grown in winter in paddy fields in off-season in Japan (Pramanik et al., 2013). The roots of vetch possess nodules which fix atmospheric N (N_2 gas) in soil and increase nitrate-N content in soil. In contrast, the non-leguminous plants like sorghum (*Sorghum bicolor*) and hey oats (*Avena sativa*) are preferred as green manure in paddocks for paddy rice cultivation. The cultivation of hairy vetch in culture vials resulted in 10-fold increase in N_2O emission, and in the presence of sorghum seedling, the N_2O emission showed nearly 3-fold increase. However, hay oats did not show any acceleration or

suppression effect on N_2O emission (Fig. 3.21).

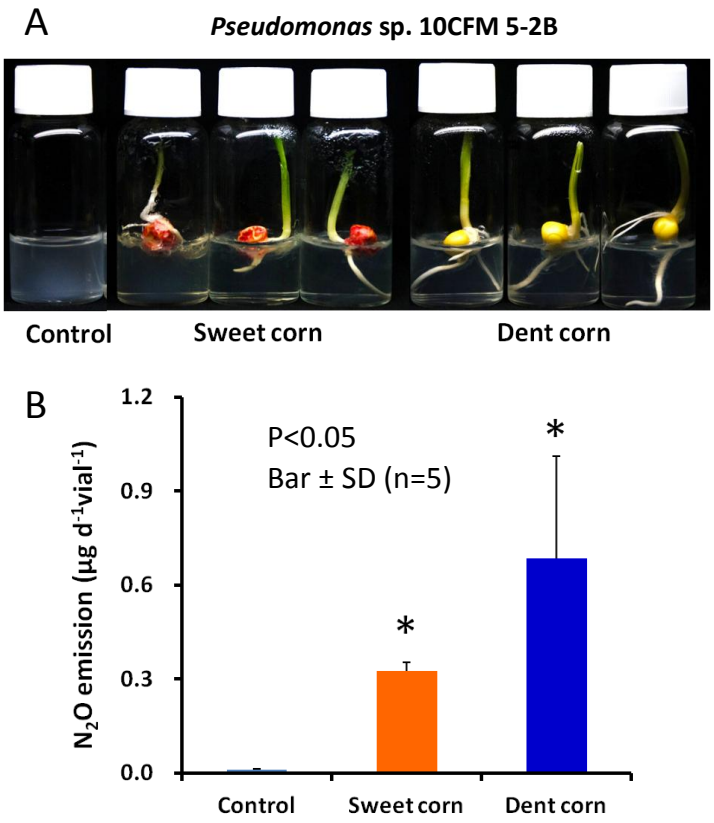


Figure 3.20 Effect of corn root exudates on N_2O production by complete denitrifier *Pseudomonas* sp. 10CFM5-2B.

The complete denitrifier *Pseudomonas* sp. 10CFM5-2B was used as the test bacterium. (A) Growth performance of sweet corn and dent corn seedling inoculated with *Pseudomonas* sp. 10CFM5-2B. Winogradsky's medium, supplemental with 0.3% (W/V) NH_4NO_3 without any carbon source, was solidified with 0.3% gellan gum. Each vial allowed germination of one seed. N_2O emittable bacteria *Pseudumonas* 10CFM5-2B was inoculated to the medium until the roots spread wildly in the medium after 1-2 days growth. The vials were covered with aluminum foil in the bottom to make sure the roots kept in dark. All the vials were kept at 25℃ in a growth chamber controlled under 12 h light-condition for 7 days. (B) N_2O production by *Pseudomonas* sp. 10CFM5-2B in the presence of corn roots exudates. Values are means ± SD (shown by error bars) (n = 5). *P < 0.05 by Student's *t*-test.

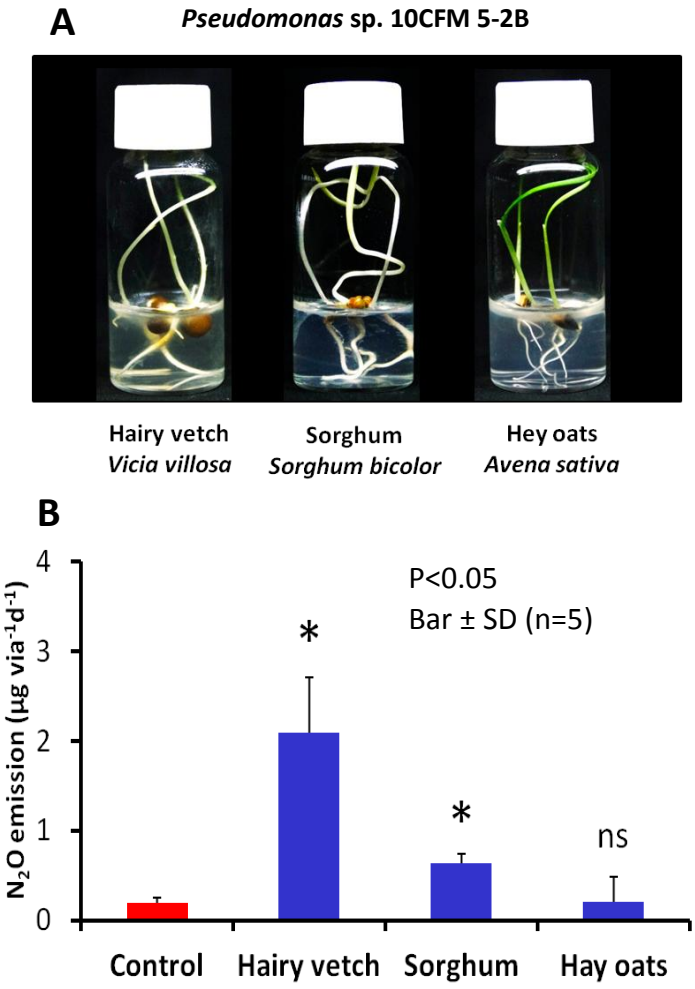


Figure 3.21 Effect of root exudates on N_2O production by complete denitrifier *Pseudomonas* sp. 10CFM5-2B.

Hairy vetch, sorghum and hay oats were used as target plants to investigate the influence of roots exudates on N_2O production by *Pseudomonas* sp. 10CFM5-2B. (A) Growth performance of hairy vetch, sorghum and hay oats roots inoculated with *Pseudomonas* sp. 10CFM5-2B. Winogradsky's medium was supplemental with 0.3% (W/V) NH_4NO_3 without any carbon source, solidified with 0.3% gellan gum. Each vial contains three seeds. N_2O emittable bacteria *Pseudumonas* 10CFM5-2B was inoculated to the medium until the roots spread wildly in the medium after 1-2 days growth. The vials were covered with aluminum foil in the bottom to make sure the roots kept in dark. All the vials were kept in 25 ℃ in the growth chamber and 12 h-light condition for 7 days. (B) N_2O production by *Pseudomonas* sp. 10CFM5-2B in the presence of each root exudate. Values are means ± SD (shown by error bars) (n = 5). *$P < 0.05$ by Student's *t*-test.

Chapter 4

Total Discussion and Conclusion

4.1 Spatiotemporal variations in N_2O emission in relation to N_2O emitting bacteria and their characteristics

4.1.1 Seasonal change of soil bacterial community in association with N_2O emission from Andisol farmland

N_2O emission from agriculture systems is related to seasonal variations of soil conditions (Jeong et al., 2012). As demonstrated in N_2O emission assay cultured in medium supplemented with NO_3^--N, N_2O emission from soil collected in April 2012 (snow melting), was higher than the soils collected in autumn 2011(Figs. 3.1 and 3.3). Seasonal variations (a combination of soil temperature, available soil moisture, nutrient levels and other potential factors) influenced the diversity of nitrifier and denitrifier populations. For example, in the farmland planted with soybeans in May 2004 and corn in May 2005, the lowest diversity of N_2O producing bacteria appeared in frozen soil in February, and rapidly increased in March, corresponding with spring thaw N_2O emission (Smith et al., 2010). In wheat field, some genera of bacteria, such as *Micrococcus*, *Arthrobacter*, and *Corynebacterium* were detected throughout the year, while *Bacillus* was found only in July. Diversity of bacterial isolate was lowest in July, and the most abundant species, *Arthrobacter oxydans*, and members of the genus *Pseudomonas* were found to reduce their cell population density in soil in July (Smit et al., 2001). In corn field, an increased diversity of genus *Burkholderia* was detected in spring, while the abundance of *Janthinobacterium* was drastically decreased. *Massillias* were detected as the dominant denitrifiers in both spring and autumn (Fig. 3.10).

Several field studies in the temperate regions have indicated that due to the freezing and thawing events in agricultural soils, N_2O emission in winter and spring

can reach between 20% and 70% of the annual budget (Van Bochove et al., 2000). The freeze-thaw events change the soil structure, such as the disruption of soil aggregates (Bullock et al., 1988), the release of aggregate-protected organic carbon (Edwards and Cresser, 1992), and the death of microorganisms, resulting in increased availability of substrate and enhanced microbial activity, leading to high amounts of N_2O emission in spring (Fig. 3.10). An increased availability of the substrates, attributed to freeze-thaw of soil, could stimulate the activity of denitrifiers in the soil. Laboratory experiments with upper soil layer of a grassland were conducted in microcosms during the entire phase of freeze and thaw, and it showed that higher levels of transcription of nitrate reductase (*napA*) and cytochrome *cd1* nitrite reductase (*nirS*) genes, just after the thawing began (Sharma et al., 2006).

In addition, a positive relationship between CO_2 and N_2O emissions was studied both by field experiments (Chu et al., 2007; Toma and Hatano, 2007) and laboratory incubation (Hashidoko et al., 2008). CO_2 fluxes from soil surface including roots in fertilizer, manure and control plots in grassland of Andisoil, were higher in spring (March to June) than in other seasons (July to February) at the same soil temperature. This suggests that there might be an increase in root respiration or heterotrophic respiration produce by fine roots (Shimizu et al., 2009; Shimizu et al., 2010). The distinction of CO_2 fluxes between different seasons associated with root respiration or heterotrophic respiration may have a great influence on N_2O emission.

4.1.2 Impact of plant species on N_2O emission

Studies in temperate ecosystems and plantations demonstrated that plant species can influence soil N_2O emission (van Haren et al., 2010). As shown in N_2O emission assay, the soil planted with corn exhibited more active N_2O production than that of pastures. Plants thus attributed to the variations of soil microbial communities in agricultural field or in terrestrial ecosystems (Figs. 3.2 and 3.4). Microorganisms in root-associated habitats (rhizosphere) may respond to the amount, composition, and spectra of root exudates, leading to the development of plant-specific microbial

communities (Wieland et al., 2001; Kowalchuk et al., 2002). However, growth conditions and developmental stage of plants may also affect its root exudation, potentially masking species-specific effect (Gransee and Wittenmayer, 2000). Because interaction between substrate-plant is involved in a highly complex environmental system that acts as a reservoir for environmental functional microorganisms, micro plants thus affect microbial processes and denitrifier communities in agricultural soil (Wu et al., 2009).

4.1.3 Nitrogen fertilizer and manure attributed to active on N_2O emission

Nitrogen fertilizer is one of the most environmentally sensitive and high impact factors in corn production. The application of chemical or organic fertilizer to soil stimulates N_2O production via the biochemical process of nitrification and denitrification (Akiyama et al., 2004). As reported, over 70% of greenhouse gas emissions in corn production are related to nitrogen fertilizer, including greenhouse gas emissions associated with nitrogen fertilizer production and soil N_2O emission (Kim and Dale, 2008). In Shizunai Livestock Farm relatively newly soil which supplied with fertilizer and manure since 2010 and collected both in autumn 2011 and spring 2012, the most active annual N_2O efflux was observed (Fig. 3.1 A, Fig. 3.3 A). Previous study on Andisol also demonstrated that the application of chemical fertilizer or manure increased N_2O emission in grassland located at Nakashibetsu, Shin-hidaka, Nasushiobara and Kobayashi (Shimizu et al., 2013). Similarly, the application of fresh cattle slurry combined with calcium ammonium nitrate mineral fertilizer induced an increase of N_2O flux during the first 4 days (Dittert et al., 2005), and N_2O emission can be affected by fertilizer type (Akiyama and Tsuruta, 2003; Akiyama et al., 2006). Furthermore, the soil supplied with fertilizer or manure also showed more active N_2O production compared with the soil without any fertilization (CC) which did not show any significant N_2O production (Figs. 3.1A and 3.3A). The organic fertilizer contains an available fraction of C compounds such as volatile fatty acids and water-soluble materials which stimulate soil nitrogen biological processes, including nitrification

(Müller et al., 2003) and denitrification (Rochette et al., 2000).

While numerous studies reported that nitrogen fertilization promotes the process of denitrification, lesser researches focus on the impact of fertilizers on the composition of denitrifying community in arable soil. The application of organic or mineral fertilizer could affect both diversity and population of the denitrifying community (Fig. 3.10), with a possible influence on N_2O fluxes (Dambreville et al., 2006b). As showed in this study, some genera, such as *Janthinobacterium, Massilia, Paenibacillus*, and *Burkholderia* were detected in soil treated with fertilizer and manure, while the diversity of *Burkholderia* and *Janthinobacterium* significantly reduced in soil without any fertilization. In contrast, Wolsing and Prieme revealed small variations in the denitrifying community which may have been caused by type of fertilizer but not by amount of fertilizer (Wolsing and Priemé, 2004). In addition, the effect of nitrogen fertilizer on both denitrifying bacteria and ammonia-oxidizing bacteria were studied in an incubation experiment, and as a result, application of high concentrations of ammonium enhanced the N_2O production and induced a shift in the soil-denitrifying community, but did not in the ammonia-oxidizing community (Avrahami et al., 2002). The changes in denitrifying community structure in soil amended with manure and fertilizer may contribute to the variations of N_2O emission with fertilization.

4.1.4 Comparison of active N_2O emitters isolated from Andisol and peat soil farmland

In this study, 10 post-harvest soil samples (collected in autumn, 2011) used for the inoculants showed active N_2O emission, and all of the four isolates that exhibited high capacities of N_2O emission were identified as *Pseudomonas* spp. (Table 3.1). In previous study on Andisol corn farmland done in the laboratory, an actinobacterium, *Streptomyces* sp. M2-0C, showed a weak N_2O emitting potential, while *Paenibacillus* sp. P1-0C (a Gram-positive bacillus of Firmicutes) and tentative *Leptothrix* sp. P2-5B (a Gram-negative rod of subdivision β-Proteobacteria) isolated from pastures of

Andisol were identified as N_2O emitters (Takeda et al., 2012). To reexamine bacterial communities showing the higher N_2O production from spring soil of relatively new corn farm with manure and chemical fertilization, we sampled the same corn farmland soils in late April 2012 just after thawing and assessed them by the culturing N_2O emission assay. We found that microbial communities from the spring soils of the Andisol corn farmland had noticeably high N_2O emission potentials in the culturing N_2O emission assay and emitted high amounts of N_2O (max. over 2.0 μg d^{-1}) (Fig. 3.3).

It is known that the area of agricultural peat soil farms in Central Kalimantan, Indonesia, is one of the most active N_2O emitting sites. Three out of 14 bacterial isolates showed N_2O emission, and the N_2O emitting *Janthinobacterium* sp. isolate A1-13 (β-Proteobacteria) exhibited 50-500 fold higher activity than N_2O emitters isolated from Andisol farmland. It was also apparent that N_2O emitting performance of soil bacteria in Andisol is distinguishable from those isolates from tropical peat soil in both quality and quantity. The N_2O-emitting ability of *Janthinobacterium* sp. A1-13 was unexpectly high, particularly in the presence of carbon sources, so that the N_2O emitter has a set of genes associated with incomplete denitrification reaction (Hashidoko et al., 2008).

The N_2O-emitting ability of *Pseudomonas* sp. 10CFM5-1B, 10CFM5-2D, and 10CMF15-2A, all likely to be incomplete denitrifiers, was comparatively higher than that of the complete denitrifier *Pseudomonas* sp. 10CFM5-2B (Fig. 3.7). Biologically, N_2O released into the atmosphere is often formed by an incomplete denitrification reaction (Firestone et al., 1980), which is attributed to the lack of nitrous oxide reductase (catalytic reduction of N_2O to N_2) in some denitrifiers (Henry et al., 2006; Richardson et al., 2009).

4.1.5 Parameters affecting N_2O emissions

N_2O is an intermediate of heterotrophic denitrification and is released in high quantities in low oxygen environments that provide sufficient NO_3^- and organic

carbon sources (Hu et al., 2013). N_2O emission is affected by many parameters such as oxygen, pH, temperature, and precipitation (Lu et al., 2006). Carbon availability also has a great impact on N_2O production (Adouani et al., 2010). All the isolated N_2O-emitting *Pseudomonas* spp. exhibited good bacterial cell growth near the gel surface in the sucrose-supplemented media, indicating that they are dependent on aerobic respiration. Addition of 0.05-0.5% sucrose as a carbon source triggered the nitrate respiration, leading to production of excessive N_2O. Organic acids produced from sucrose by the bacterium may assist N_2O production due to maintaining acidity that inhibits N_2O reduction (Hashidoko et al., 2008). In this study, 0.05% sucrose was generally used as the carbon source to ensure the normal growth of N_2O emitters, to avoid their excessive growth, and to investigate the optimum pH for N_2O emitters (Fig. 3.5).

It is reported that the N_2O/N_2 product ratio in denitrification is higher in acidic than in alkaline soil (Šimek and Cooper, 2002; Bergaust et al., 2010), because of the pH effect on the transient accumulation of N_2O production by denitrification in soil (Liu et al., 2010; Dorsch et al., 2012). There is an ample evidence that an acidic soil in Germany (pH 5.4) accumulated much more N_2O than neutral soil in Finland and Sweden (pH 6.0 and 7.1, respectively) (Holtan-Hartwig et al., 2002). Thus, N_2O production from soil is generally reduced when the pH shifts to the optimum range for N_2O emitters. This trend has also been reported in several types of soil bacterial microbiota (Martikainen and Deboer, 1993).

As shown in Fig. 3.8, N_2O emission from active bacteria isolated from the post-harvest soils is significantly affected by pH. The pH values of the medium decreased slightly after inoculating with N_2O emitters and incubation for 7 days, but the values remained in the neutral region, even in the medium containing 0.05% sucrose (Fig. 3.8). *Pseudomonas* sp. 10CFM5-1B exhibited maximum N_2O production at pH 5.5-6.7, a relatively wide optional pH range, while *Pseudomonas* sp. 10CFM5-2D showed a relatively narrow range from 6.0 to 6.2 optimum for N_2O production (Fig. 3.8). The neutrophilic-like behaviors of these denitrifiers suggest that the N_2O emitters from the post-harvest soil sampled in autumn are highly root

associated (Philippot et al., 2007; Philippot and Hallin, 2011). Indeed, many plants can maintain rhizosphere pH in neutral regions even in strongly acidic soils (Hashidoko et al., 2005; Philippot and Hallin, 2011); therefore, neutrophilic rhizobacteria are probably dominant in the post-harvest soils rich in plant root residues.

4.2 Effect of C- and N-sources on the diversity of eubacteria and N_2O emission

The purpose of this study was to investigate the effects of carbon and nitrogen sources on N_2O emission characteristics and diversity of soil microbial communities. To identify the main source of emitted N_2O, several incubation experiments were carried out with modified Winogradsky's gellan gum medium supplemented with alternative carbon and nitrogen sources. Community composition of the denitrifying bacteria under different carbon and nitrogen sources were analyzed using DGGE technique.

4.2.1 Seasonal variations in diversity of denitrifier related to N_2O emission

Soil contains an enormous number of living organisms including bacteria, archaea, protozoa, fungi, nematodes and arthropods. Both nitrifiers and denitrifiers in soil play a key role in nitrogen cycling. Bacteria capable of denitrification can be easily isolated from sediment, soil and aquatic environments. Recent studies have shown that some species of various genera such as *Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium* and *Hyphomicrobium* as well as *Pseudomonas* species are responsible for denitrification in soil (Lim et al., 2005).

Studies have shown that the composition of the nitrifying and denitrifying communities is important in regulating the N_2O flux from soil and other ecosystems (Smit et al., 2001; Avrahami and Conrad, 2005). Soil organisms are naturally active

during certain times of year. Most are active when the soil is warm and moist, like during spring showed relatively high diversity and intensity of denitrifier *Burkholderia* sp. in soil 10CFM15 and CK15 leading to active N_2O emission (Fig. 3.10). During autumn, rain provide moisture to the soil while it is still warm, soil organisms may maintain their activity. As the soil cools in the late autumn, many soil microorganisms turn into dormant. As shown in the result (Fig. 3.10), the abundance of denitrifer *Burkholderia* sp. significantly decreased in CK5 and CK15 in autumn. Studies in site of Ontario, Canada, revealed that diversity of the populations of nitrifying and denitrifying bacteria and archaea was lowest in February, in frozen soils, and rapidly increased in March, along with spring thaw that activated N_2O emission (Smith et al., 2010).

Seasonal variation (a combination of soil temperature, available soil moisture, nutrient levels and other potential factors) had the largest influence on the diversity of nitrifier and denitrifier populations. Microbial N_2O releasing during the course of thawing of the soil was investigated in a model experiment focusing on denitrification, since freeze-thaw has been shown to cause significant changes of soil in physical and biological conditions, including a burst of N_2O. The increase in denitrification after thawing may be attributed to the diffusion of organic substrates newly provided to denitrifiers from disrupted soil aggregates, leading to an increase in microbial activity to emit N_2O (Sharma et al., 2006).

Therefore, it would be reasonable to conclude that N_2O emissions in autumn and spring is associated with seasonally different community structure in soil.

4.2.2 N_2O emission and composition of denitrifying bacterial community respond to fertilization

Denitrification is a significant contributing process to emissions of N_2O, which is involved in destruction of the stratospheric ozone layer and global warming. The nitrate-reducing bacteria comprise a large group of phylogenetically unrelated microorganisms. Since denitrification can result in losses of added nitrogen fertilizers

from agricultural soil, numerous studies showed that nitrogen fertilizer promotes development of denitrification community and acceleration of denitrification process.

In our incubation experiment, soil applied with chemical fertilizer and manure exhibited (Fig. 3.10) relatively high diversity of denitrifier community than the control (without any fertilization), expecially the dominant species of *Burkholderia* which contributes to active N_2O emission. The effect of nitrogen fertilization on both denitrifying bacteria and ammonia-oxidizing bacteria were studied in an incubation experiment (Avrahami et al., 2002), showing that addition of high concentration of ammonia induced a drastic shift of the soil denitrifying community structure (Fig. 3.12). Temporal and spatial variation of denitrifying bacterial communities at site where received mineral fertilizer (60 and 120 kg N $ha^{-1}year^{-1}$) and cattle manure (75 and 150 kg N $ha^{-1}year^{-1}$) were analysed, showing different communities of *nirK*-containning denitrifying bacteria (Enwall et al., 2005). In a field experiment, small variations in the denitrying community were observed, in which bacterial community structure may have been affected by type of fertilizer but not amount of fertilizer (Wolsing and Priemé, 2004). Even in long-term field experiments, both abundance and activity of ammonia oxidizers were affected by the nitrogen fertilization (Phillips et al., 2000; Webster et al., 2002). Furthermore, amendment of total bacterial community structure in soil by application of manure or ammonium nitrate has been reported (Peacock et al., 2001). Taken together, land management, particularly chemical fertilization and manure input, is one of the most important factor for N_2O emission form agricultural soil, including Andisol in cold-temperature zone.

4.2.3 Soil sources influence patterns of denitrifying communities

The diversity of microorganisms and N_2O production can be influeced by many parameters such as substrate concentrations, C/N ratio, nitrite accumulation, and NO concentration. Among these parameters, chemical properties of the carbon source have a large effect on the N_2O production (Adouani et al., 2010) .

In soil incubation with different supplementation assay, addition of sucrose and alternative nitrogen source exhibited significant accelaration of N_2O emission, but from the DGGE profiles, there was no obviously differences because of high diversity of microbial communities in the soil (Fig. 3.9). In contrast in soil suspension culture assay, addition of carbon source sucrose as 0.05% increased abundance and diversity of denitrifying bacteria *Burkholderia* sp. (Fig. 3.11), and the similar results were also shown in other modification of the media (Fig. 3.13). The N_2O releasing activity of the soil denitrifier community clearly responded to the addition of 0.05% sucrose, resulting in a higher N_2O emission rate than the control (without sucrose). Abundance of the total bacterial community increased in soil amended with 1,000 mg C kg^{-1} of soil as glucose or red clover tissue, however, both simple (glucose) and complex (plant residues and liquid manures) C sources applied at a rate of 250 mg C g^{-1} soil (Miller et al., 2008) or addition of 150 mg C g^{-1} soil for one month as artificial roots exudates (Henry et al., 2008) did not cause changes in the abundance of the total bacterial community.

On the other hand, this study showed that the soil nitrifier population represented by DGGE profiles showed a drastic change during incubation of the soil culture in alternative nitrogen sources. As a result shown in Fig. 3.13, abundance of an ammonia-oxidizing bacterium *Arthrobacter* sp. was obveriouly increased under the presence of ammonia as N_2O substrates to cause of lower N_2O production than the control. Therefore, the response of the N_2O releasing activity was probably due to the major change in the ammonia-oxidizing population. This result is agreeable with other studies, both in the laboratory (Schuster and Conrad, 1992) and field (Müller et al., 1998) studies observed as an increased contribution of nitrification to N_2O emission in correlation with increasing ammonium concentration. High diversity of *amoA* sequences from the different ammonium treatments (58 and 395 μg of NH_4^+-N g [dry weight] of $soil^{-1}$) was observed, affiliated with five *Nitrosospira* and one *Nitrosomonas* (Avrahami et al., 2002). However, other bacterial populations, such as denitrifiers affected by the addition of ammonium to cultured medium, contributed to N_2O emission from the soils leading to decreased intensity of *Burkholderia* (Fig.

3.13C). Similalily to the results for the population density of ammonia-oxidizers, significant changes were detected in the bacteria community from the untreated soil and after treatment with ammonium, nitrogen-fixing bacteria *Clostridium* was in high population in the untreat soil (Fig. 3.12 C).

The results showed that rates of N_2O emission from the soil are positively correlated with carbon and nitrogen sources. The contribution of carbon and nitrogen sources to N_2O production increased by ammonia oxidation which provided the electron acceptor for denitrification. Addition of sucrose accelerated the emergence of saprophytic bateria and fungi, while in the presence of alternative nitrogen sources, particularly ammonia, stimulated the emergence of anaerobes and ammonia oxidizers *Arthrobacter*, while addition of nitrate increased the diversity and abuncance of denitrifying bacteria *Burkhoderia* spp.

4.3 Effect of methyl viologen dichloride and other chemicals on nitrous oxide (N_2O) emission and repression by pseudomonad denitrifiers isolated from corn farmland soil

4.3.1 Repression effects of chemical compounds on N_2O emission by pseudomonads

The aim of this study was to demonstrate the inhibitory effects of chemical pesticides toward soil denitrifiers of root-associating saprophytic pseudomonads (Philippot et al., 2007; Philippot and Hallin, 2011). Acetylene is the most potent chemical that selectively inhibits nitrous oxide reductase (N_2OR) (Matsubar and Mori, 1968; Balderston et al., 1976), while other chemicals including azide anion, thiocyanate, carbon monoxide, and cyanide, are also significant N_2OR inhibitors (Kristjansson and Hollocher, 1980). However, those selective inhibitors are highly toxic to mammals and difficult to handle towing to their instability or gaseous states. Denitrification inhibitors hydroxamine (against nitrate reductase) (Kučera and Skládal, 1990), methanol, and allylthiourea (both in the anammox process) (Jensen et al., 2007)

have also been reported, but are not applicable to agricultural farm soils.

In a preliminary study, we had hypothesized that methyl viologen dichloride (Paraquat®) would accelerate N_2O production, as it is a known competitive substrate of N_2OR (Kristjansson and Hollocher, 1980). However, this herbicide completely repressed N_2O production of pseudomonads at 5 μM (Fig. 3.14). It has been reported that methyl viologen dichloride non-selectively inhibits any redox enzymes associated with the denitrification process (Day et al., 1999). However, at 5 μM of the herbicide, the bacterial cells still survived in the cultured medium, and hence it is more likely that methyl viologen dichloride rather inhibit some specific enzymes and one of denitrifcation associated genes is probably one of them. Even at 1 μM, equivalent to approximately 200 μg L^{-1}, this herbicide resulted in 50% inhibition of N_2O production. Allowable dose limit of residual Paraquat in crops are 0.5 ppm (2 μM), which is probably a similar level of the adsorbed Paraquat® in the soil. Although Paraquat® is not currently approved as a herbicide in Japan, this chemical continuously used widely in developing countries, and likely contributes to N_2O repression particularly in plantation soil reclaimed from tropical peat swamp forests.

Conversely, 6-methoxy-2-benzoxazolone (MBOA), an antifungal secondary metabolite from corn, also repressed pseudomonad denitrification at 10 μM; however, its inhibitory effect on N_2O emission was not significant at 5 μM (Fig. 3.17). In contrast, the structurally similar benzoxazoline derivatives 2-benzoxazolinone (BOA) and 1-hydroxy-1*H*-benzotriazole (HOBt) were both inactive against N_2O emission by the denitrifying pseudomonads. At concentrations greater than 150 μM, both BOA and HOBt exhibited a remarkable repression of N_2O production, but the effects of BOA and HOBt on the repression of N_2O emission were mainly due to the repression of bacterial cell growth, not as pin-point inhibition of enzymes associated with N_2O production (Fig. 3.16).

4.3.2 Acceleration effects of herbicides on N_2O emission by *Pseudomonas* sp. 10CFM5-2B

Other herbicides, such as simazine, trifluralin, and amitrol, rather accelerated N_2O emission by *Pseudomonas* sp. 10CFM5-2B at 2 or 10 μM concentrations. Particularly, 2 μM amitrol led to a 2.5-fold increase in N_2O emission, suggesting that farm soil sprayed with amitrol became an active N_2O efflux source (Fig. 3.18). As another study, methyl parathion stimulated denitrificaiton as it increased the NO_3^- reduction, the concentration of NO_2^-, and the emission of N_2O and N_2 (Blanco-Jarvio et al., 2011). Methyl parathion can serve as a C-substrate and electron donor thereby increasing NO_3^- reduction and consequently emission of N_2O and N_2 (Ramanathan and Lalithakumari, 1999). The possibility of the selective inhibition of N_2OR by amitrol in contrast to other reductoxidases in the denitrification process should be investigated further.

4.4 Interactions between root exudates and N_2O production by *Pseudomonas* sp. 10CFM5-2B

4.4.1 Plant species affect denitrifier activities associated with N_2O emission

Several studies have reported that the presence of plant roots can stimulate the denitrification (Stefanson, 1972; Vinther, 1984; Svensson et al., 1985). This stimulation was attributed to both root respiration to decrease micro-environmental oxygen contents and the exudation of several organic compounds by the roots to be utilized as carbon sources (Leif Klemdetsson, 1987). Effects of plant species have mainly focus on denitrifier activity rather than denitrifier community structure, and are due to the differences in quality and quantity of organic compounds from the roots (Henry et al., 2008; Philippot et al., 2008). Several studies reported that higher denitrification rates were observed in the rhizosphere of legumes compared to other plants, the denitrification rates of nitrogen-fixing Lucerne ley were higher than those of the barley and grass ley (Svensson et al., 1991; Kilian and Werner, 1996). Similar

results were also shown in Fig. 3.20, in which nitrogen-fixing leguminous plant hairy vetch exhibited the most active acceleration of N_2O emission than sorghum and hay oat. Denitrification activities of grass tufts among three species (*Holcus lanatus*, *Arrhenatherum elatius* and *Dactylis glomerata*) also showed the significant differences in plant species (Patra et al., 2006).

Roots are likely to affect denitrification through chemical and physical modifications of the surrounding soil known as rhizosphere, such as concentrations of NO_3^-, oxygen, and available organic carbon sources (Woldendorp, 1962). Some other factors are likely to affect denitrification indirectly (Wollersheim et al., 1987). For example, changes in soil-water content can alter the concentrations of oxygen in soil. Three ways were found for the capablity of lowering soil oxygen concentrations of roots, 1) roots respiration known as a potential sink for oxygen; 2) organic carbon released into soil from the roots in form of soluble exudates and organic materials; 3) fine roots penetrating into soil to increase soil compaction.

In this study, the primary driver of rhizosphere bacteria *Pseudomonas* sp. development is the release of plant-derived low molecular weight organic compounds into the medium, with an increased total C and soluble organic C which accelerated denitrification rates and N_2O emission (Baggs and Blum, 2004). However, the contradictory results have also been reported: the effect of the released organic compounds by roots on denitrification. Haider et al. have reported that root exudates could not provide sufficient organic compounds to the process of denitrification (Haider et al., 1987), or that root-derived organic compounds were rapidly immobilized or mineralized by microorganisms in the rhizosphere, leading to little influence on denitrification (McCarty and Bremner, 1993). Addition of artificial root exudates or mucilage to soil without plants stimulated nitrate reduction or denitrification activity along with increases in the range of those observed in the whole plant tissues (Henry et al., 2008).

4.4.2 Composition of bacterial functional communities associated with N_2O emission affected by root exudates

While effect of plant species on activity of denitrifier communities have widely been reported, few studies focus on effect of a plant species on denitrifier community structures. The diversity of denitrifying bacteria was different between soils with and without maize, and a plant-dependent enrichment of *Agrobacterium*-related denitrifiers has been observed in a modification of the denitrifying community structure between maize planted and bulk soil (Cheneby et al., 2004). Addition of artificial root exudates did not show any significant differences in the structure or the density of nitrate reducer and denitrifier communities, even though nitrate reduction and denitrification activity were strongly stimulated (Henry et al., 2008). Individual species of non-leguminous plants directly influenced the composition of denitrifier communities, e.g., through their root exudates. In addition, the genetic structure of the nitrate-reducing microbial community in soil below grass tufts dominated by *Arrhenatherum elatius*, *Dactylis glomerata*, and *Holcus lanatus* (all of the grass species are belong to Poaceae) was dependent on the plant species (Patra et al., 2006). The *nirK*-type denitrifiers were also found in the rhizospheres of three legume crops (*Vicia faba, Lupinus albus,* and *Pisum sativum*), and the diversity and composition of *nirK* transcripts were influenced by the plant species (Sharma et al., 2005).

4.5 General conclusion

In conclusion, it was discovered in this thesis the active N_2O emitters, both *nosZ*-negative and *nosZ*-positive *Pseodomonas* spp. denitrifiers, were isolated from post-harvest Andisol farm soil. The *nosZ*-negative bacterial isolates from post-harvest soil showed greater acceleration of the N_2O production than isolates obtained from grasslands. Corn farm soils collected in spring 2012 showed higher N_2O-emitting capabilities than those collected post-harvest, attributed to the high content of saprophytic incomplete pseudomonad denitrifiers. An active supply of organic

substances from corn roots and sufficient fertilization of acidic soils could assist active N_2O emission via *Pseudomonas* spp. denitrifiers. It was thus likely that seasonal change of the soil microbial communities in the Andisol farmland is highly related to development of crop root system along with active N_2O emission from the soils.

Some commercial herbicides, a representative antimicrobial secondary metabolite of corn, 6-methoxy-2-benzoxazolone (MBOA), and *N*-heterocyclic compounds structurally related to MBOA were examined their effects on N_2O-emitting soil bacteria. It was found that methyl viologen dichloride (Paraquat®) at 2 μM significantly repressed N_2O emission by the active denitrifying bacteria. MBOA also repressed pseudomonad denitrifiers at a concentration of 10 μM. Other herbicide such as simazine and amitrole accelerated N_2O emission by *Pseudomonas* sp. 10CFM5-1B at 2 μM or 10 μM. For the perspective of regulation of N_2O emission by N_2O-emitting bacteria, the insight into mechanism of this reaction especially in the area of gene expression and enzyme activity will lead to biocontrol agents. In near future, development of environmental-friendly herbicides and other agricultural chemicals that minimize N_2O emission will promote the sustainability of the agricultural soil.

For controlling of N_2O emission by soil management in agricultural ecosystem particularly in fertilization, the effect of C- and N-sources on Andisol microbial community structures and N_2O emission were investigated. The findings showed the rates of N_2O emission from the soil are positively correlated with carbon and nitrogen sources, and both denitrifiers and unculturable ammonia-oxidizing bacteria in soil contribute to active N_2O emission. These findings may further serve as potential targets for directly development of controlling approaches to regulation of N_2O emission in agricultural ecosystem.

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