

2005

## Nitrate reduction and methane formation as influenced by iron-centered intermediate redox processes in rice soils

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**NITRATE REDUCTION AND METHANE FORMATION AS INFLUENCED BY  
IRON-CENTERED INTERMEDIATE REDOX PROCESSES IN RICE SOILS**

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Oceanography and Coastal Sciences

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May 2005

## **ACKNOWLEDGMENTS**

My sincere thanks go to my major professors Dr. William H. Patrick Jr. and Dr. Robert P. Gambrell for their guidance and encouragement in my study. My sincere thanks also go to other committee members Dr. Harry H. Roberts, Ralph J. Portier and Jaye E. Cable, for their time and suggestions, and to Dr. Wayne H. Hudnall and Dr. Lewis A. Gaston, for serving as the representative of the Graduate School Dean in my general and final exams, respectively.

I would like to extend my grateful acknowledgment to Dr. Kewei Yu, Dr. Ron Delaune, Dr. Chuck Lindau, and everyone else from the Wetland Biogeochemistry Institute, Louisiana State University for all kinds of help in my study program. The funding support from the United States Department of Agriculture is appreciated. Finally, I thank my wife Rui and our daughter Yiming, who gave me care and encouragement through the whole period of study.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	ii
ABSTRACT.....	v
CHAPTER	
1. INTRODUCTION.....	1
Biochemical Redox Reactions.....	1
Anaerobic Redox Processes.....	1
Ecoenvironmental Aspects of Iron Redox Processes.....	6
Research Objectives.....	12
2. EFFECT OF MICROBIAL IRON REDUCTION ON DENITRIFICATION AND METHANOGENESIS IN ANOXIC SOIL SLURRIES.....	14
Introduction .....	14
Materials and Methods.....	16
Results.....	19
Discussion.....	25
Conclusions.....	28
3. TEMPORAL AND VERTICAL VARIATIONS OF NITROUS OXIDE AND METHANE PRODUCTION AFFECTED BY IRON-CENTERED INTERMEDIATE REDOX PROCESSES IN A FLOODED SOIL.....	30
Introduction .....	30
Materials and Methods.....	32
Results.....	34
Discussion.....	40
Conclusions.....	44
4. FATE OF NITRATE IN A FLOODED RICE SOIL AFFECTED BY POOL OF ALTERNATIVE ELECTRON ACCEPTORS.....	46
Introduction .....	46
Materials and Methods.....	48
Results.....	50
Discussion.....	54
Conclusions.....	57

5. MICROBIAL ACTIVITY IN DIFFERENT REDOX ZONES OF A FLOODED SOIL.....	59
Introduction .....	59
Materials and Methods.....	61
Results.....	62
Discussion.....	68
Conclusions.....	69
6. BIOELECTROCHEMICAL NITRATE REMOVAL FROM WATER USING FLOODED SOIL AS A REDUCTANT SOURCE.....	71
Introduction .....	71
Materials and Methods.....	73
Results.....	76
Discussion.....	79
Conclusions.....	82
7. EFFECTS OF FERRIC IRON AMENDMENT ON NITRATE PERCOLATION AND EMISSIONS OF NITROUS OXIDE AND METHANE IN A RICE SOIL.....	84
Introduction .....	84
Materials and Methods.....	86
Results.....	89
Discussion.....	94
Conclusions.....	96
8. SUMMARY AND FUTURE RESEARCH RECOMMENDED.....	97
Summary .....	97
Conclusions.....	100
Future Research Recommended.....	101
REFERENCES .....	102
VITA.....	112

## ABSTRACT

Rice fields are a major source of the greenhouse gases methane ( $\text{CH}_4$ ) and nitrous oxide ( $\text{N}_2\text{O}$ ) and contribute to nitrate ( $\text{NO}_3^-$ ) pollution in waters. Ferric iron ( $\text{Fe}^{3+}$ ) and manganic manganese ( $\text{Mn}^{4+}$ ) are two intermediate alternative electron acceptors (AEAs) capable of regeneration in freshwater soils. In this investigation, the influences of iron-centered intermediate redox processes on  $\text{NO}_3^-$  reduction and  $\text{CH}_4$  formation in rice soils were studied using soil slurries, soil columns, and potted rice.

Reduction of  $\text{Fe}^{3+}$ -centered intermediate AEAs was mainly mediated by obligate anaerobes relying on fermentation products. Ferric iron reducers are bioelectrochemically active, supporting bioelectricity generation through a fuel cell process from the flooded soil coupled to the reduction of  $\text{O}_2$  or  $\text{NO}_3^-$  in the overlying water. As a major electron accepting process in anaerobic carbon decomposition,  $\text{Fe}^{3+}$  reduction stimulated  $\text{N}_2\text{O}$  production but had little influence on overall  $\text{NO}_3^-$  reduction in the homogenized soil slurries under near-neutral pH conditions. In the flooded soil column and pot experiments, intensification of iron-centered intermediate redox processes under amendments of iron and/or manganese oxides changed the fate of  $\text{NO}_3^-$  in the overlying water, decreasing heterotrophic denitrification and increasing  $\text{NO}_3^-$  percolation and  $\text{N}_2\text{O}$  emission. Ferric iron reduction competitively suppressed methanogenic activity in the homogenized soil slurries. The diffusion of the stronger oxidants  $\text{O}_2$  and  $\text{NO}_3^-$  controlled temporal and vertical variations of iron-centered intermediate redox processes, which subsequently controlled temporal and vertical variations of methanogenic activity in the flooded soil

columns. In the pot experiment,  $\text{Fe}^{3+}$  reduction had small effect on  $\text{CH}_4$  emission in the early season when  $\text{CH}_4$  emission was low but effectively reduced  $\text{CH}_4$  emission after midseason drainage intervals through  $\text{Fe}^{3+}$  regeneration. The roles of iron-centered intermediate redox processes need to be considered in the evaluation and predication of  $\text{NO}_3^-$  reduction and  $\text{CH}_4$  formation in rice fields.

# **CHAPTER 1**

## **INTRODUCTION**

### **Biochemical Redox Reactions**

Every organism obtains energy from light or inorganic and organic substances through biochemical reduction and oxidation (redox) reactions to synthesize adenosine triphosphate (ATP) for growth and reproduction. All organisms are autotrophs or heterotrophs, according to their pathways of energy harvest. Autotrophs, including phototrophs and chemoautotrophs, obtain energy from redox reactions of inorganic substances with or without need of light. Heterotrophs, including animals and most microorganisms, obtain energy from organic substances. Nevertheless, the boundaries between autotrophs and heterotrophs are not always clear for microorganisms. When organic substances are not available, some heterotrophs become chemoautotrophs harvesting energy from inorganic substances.

### **Anaerobic Redox Processes**

Anaerobic redox processes depend on oxidants other than  $O_2$ , namely alternative electron acceptors (AEAs) as the terminal electron acceptor. Both inorganic and organic substances can be used as an AEA. The common inorganic AEAs include  $NO_3^-$ ,  $Mn^{4+}$ ,  $Fe^{3+}$ ,  $SO_4^{2-}$ , and  $CO_2$ . Many other elements such as  $U^{6+}$  and  $Se^{6+}$  can also be used as AEAs (Lovley et al., 1991; Lovley, 1993; Haveman and Pedersen, 2002). Many quinone-containing organic substances are reduced as AEAs (Fredrickson et al., 1998) for anaerobic respiration. The availability and dynamics of the common inorganic redox



species are more important in redox processes and carbon mineralization in anaerobic environments.

### **Sequential Reduction of Alternative Electron Acceptors**

Although some redox reactions such as  $\text{Fe}^{2+}$  oxidation with nitrite may happen chemically, redox reactions in anaerobic environments are largely microbially mediated with AEAs being sequentially reduced in the order of  $\text{NO}_3^-$ ,  $\text{Mn}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$  and  $\text{CO}_2$  (Patrick and DeLaune, 1972; Froelich et al., 1979; Reddy et al., 1989). The reduction of one AEA precedes the reduction of another AEA for three reasons. First, the reduction of the preceding AEA (i.e.,  $\text{Fe}^{3+}$  before  $\text{SO}_4^{2-}$ ) yields more energy and is preferred by anaerobes. Secondly, energy sources are used so efficiently in the reduction of the preceding AEA that their levels are too low to be able to simultaneously support the reduction of the following AEAs (Lovely and Goodwin, 1998). Thirdly, the preceding AEA in some cases is toxic to microbes capable of using its following AEAs. For example,  $\text{NO}_3^-$  and its intermediate products NO and  $\text{N}_2\text{O}$  are toxic to methanogens (Chidthaisong and Conrad, 2000). When  $\text{NO}_3^-$  is present, even a high level of acetate cannot be used for  $\text{CH}_4$  production. In general, the closer two AEAs are in the sequence, the less toxic is the preceding AEA to the reduction of the following AEA. For example,  $\text{SO}_4^{2-}$  is much less toxic than  $\text{NO}_3^-$  to methanogens (Chidthaisong and Conrad, 2000).

Among these three mechanisms, the thermodynamic principle of energy yield is believed to be most important and is applied as the base for modeling of microbially driven redox processes (VanCappellen and Wang, 1996; Park and Jaffe, 1996; van

Bodegom and Scholten, 2001). In addition, some microbes, including fermenting bacteria, some  $\text{SO}_4^{2-}$  reducers and  $\text{CH}_4$  producers may reduce some metals such as  $\text{Fe}^{3+}$  but not couple energy yield to support growth (Coleman et al., 1993; Bond and Lovley, 2002). Such redox reactions may not be regulated by the above three mechanisms but a physiological response for de-toxicity.

### **Overlapping of Redox Processes**

While the sequential reduction of AEAs has been found to be largely true in homogenized environments such as soil suspensions, it is largely obscured in natural environments (Gao et al., 2002). Different anaerobic redox processes occur simultaneously on a wide range of temporal and spatial scales. There are a number of reasons for such overlapping. The first one is soil/sediment heterogeneity. Various sizes of particles and other soil components, including organic matter are heterogeneously distributed, providing microsites suitable for various functional microbial groups. The bioavailability and distribution of AEAs are also heterogeneous. Among the common inorganic redox species,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$  and  $\text{CO}_2$  are soluble while  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  are largely solid and capable of recycling and redistribution (Frenzel et al., 1999; King and Garey 1999; Ratering and Schnell 2000). Direct contact is required for microbes to transfer electrons to these solid electron acceptors but an exception occurs when alternative mechanisms such as use of electron-shuttling substances are adopted (Straub et al., 2001; Luu and Ramsay, 2003). The presence of aquatic plants and bioturbation of fauna additionally create heterogeneous aerobic/anaerobic interfaces in soils and sediments. A

high excess of electron donors relative to electron acceptors also cause simultaneous occurrence of different redox processes (Roy et al., 1997; Yu and Patrick 2003).

Microbial species responsible for specific redox processes also tend to overlap. There is no clear distinction between  $\text{NO}_3^-$  reducers,  $\text{Fe}^{3+}$  reducers,  $\text{SO}_4^{2-}$  reducers, and  $\text{CH}_4$  producers in that many species of microbes that reduce other AEAs are capable of  $\text{Fe}^{3+}$  reduction. Many  $\text{NO}_3^-$  reducers are  $\text{Fe}^{3+}$  reducers. Even some  $\text{SO}_4^{2-}$  reducers and methanogens can reduce  $\text{Fe}^{3+}$  (Coleman et al., 1993; Lovley et al., 1993). Due to the prevailing ability of a large number of microbial species to use  $\text{Fe}^{3+}$  as an electron acceptor, it has been suggested that  $\text{Fe}^{3+}$  reducers are very important in the origin of life on the earth (Vargas et al., 1998).

### **Dissimilatory Microbial Reduction of Iron and Manganese**

Iron and manganese represent the 4<sup>th</sup> and 12<sup>th</sup> most abundant elements, respectively, in the Earth's crust (Horne, 1978). Both of these transition metals are not only essential trace elements for life but also important as intermediate AEAs in anaerobic carbon mineralization. Most iron compounds are particulate and incapable of microbial reduction. Still, due to its abundance,  $\text{Fe}^{3+}$  is normally the dominant AEA in freshwater soils (Yao et al., 1999). More importantly,  $\text{Fe}^{3+}$  is capable of regeneration. Rapid iron redox cycling around aerobic/anaerobic interfaces of wetland and aquatic environments further increases the importance of microbial  $\text{Fe}^{3+}$  reduction in anaerobic carbon decomposition. Even in marine sediments where  $\text{SO}_4^{2-}$  reduction is normally the dominant pathway for anaerobic carbon mineralization, microbial  $\text{Fe}^{3+}$  reduction may contribute substantially to

anaerobic carbon decomposition due to intensive iron redox recycling (Aller et al., 1996; VanCappellen and Wang, 1996).

Microbial reduction of  $\text{Mn}^{4+}$  is normally mediated by microorganisms which are also capable of  $\text{Fe}^{3+}$  reduction (Lovley et al., 2004). Since manganese is normally less abundant than iron (Horne, 1978) and  $\text{Mn}^{2+}$  reoxidation is much slower than  $\text{Fe}^{2+}$  reoxidation at oxic-anoxic interfaces (Stumm and Morgan, 1981),  $\text{Mn}^{4+}$  reduction is generally less important than  $\text{Fe}^{3+}$  reduction in anaerobic carbon decomposition. The importance of microbial reduction of other intermediate AEAs such as  $\text{As}^{5+}$  is even smaller. As such,  $\text{Fe}^{3+}$  reduction is of central importance among all intermediate redox processes occurring after the depletion of  $\text{NO}_3^-$  and before the initiation of  $\text{SO}_4^{2-}$  reduction and  $\text{CH}_4$  formation.

A number of factors hamper quantitative approaches to estimation of microbial  $\text{Fe}^{3+}$  reduction in anaerobic environments. Iron compounds have two oxidation states but have too many structurally different forms differing in solubility and bioavailability. Dissolution and precipitation of iron compounds are associated with many inorganic and organic substances, such as metals, sulfides and humic substances. Because of these complex interactions, the isotopic approach, a powerful approach to quantification of N, S and C cycling, is not applicable for quantification of microbial  $\text{Fe}^{3+}$  reduction (Roden and Lovley, 1993). Sampling of iron compounds under field conditions and quantifying the different compounds is also difficult due to their heterogeneous distribution and dynamic transformations. Reducible iron content inferred from direct extraction or

extraction after a period of anaerobic incubation (Lovley and Phillips, 1986; van Bodegom et al., 2003) has been used to assess the potential/scale of microbial  $\text{Fe}^{3+}$  reduction in anoxic soils and sediments. The influences of soil/sediment properties on the reliability of these extraction approaches deserve further investigations. A quantitative approach to estimation of microbial  $\text{Fe}^{3+}$  reduction in field conditions under rapid redox cycling is yet to be found.

### **Ecoenvironmental Aspects of Iron Redox Processes**

Iron redox cycling directly or indirectly interacts with many processes including almost all other major redox processes. Due to its close interactions with so many processes, iron redox cycling plays a key role in most anaerobic environments. Iron redox processes are a major factor shaping geochemistry of ancient environments. Formation and distributions of iron compounds, such as pyrite, are important in interpretation of modern and ancient environments (Danielson et al., 1992; Marnette et al., 1993). In modern times, iron redox processes play a number of important ecoenvironmental roles including organic pollutant degradation, metal mobilization/immobilization in subsurface environments and  $\text{NO}_3^-$  and  $\text{CH}_4$  biogeochemistry in wetland soils, etc. (Lovley, 1991; Lovley et al., 1994; Roden and Wetzell, 1996; Yao et al., 1999; Senn et al., 2002). In addition, iron-reducing bacteria, with an ability to use an electrode as the electron acceptor, are involved in bioelectricity generation from anoxic sediments preventing or decreasing the formation of  $\text{CH}_4$  (Rabaey et al., 2003) while coupling to the reduction of oxidants including nitrate (Gregory et al., 2004).

## **Organic Pollutant Degradation and Metal Stability in Subsurface Environments**

The redox state of shallow groundwater/subsurface soils is maintained by the relative rates of  $O_2$  and organic carbon inputs, microbial carbon respiration and the availability of the various AEAs. Oxidized iron and manganese minerals provide significant oxidative capacity to buffer groundwater redox quality against the input of organic carbon in the absence of oxygen. However, at point-source pollution sites, e.g. oil spill sites, electron acceptors are normally in short supply, especially after the injection of available carbon to stimulate growth of microorganisms for organic pollutant degradation. The efficiencies of use of  $O_2$  and  $NO_3^-$  as electron acceptors are low in this situation due to the difficulties in confining them within the targeted zones. Chelated  $Fe^{3+}$  has been used as an AEA to stimulate organic pollutant degradation (Lovley et al., 1994). The  $Fe^{2+}$  produced from  $Fe^{3+}$  reduction may be collected in a downgradient well for recycling.

Similarly, since redox cycling of many other elements such as U is closely associated with iron redox cycling,  $Fe^{3+}$  reduction or regeneration also controls the dissolution/precipitation of these elements (Lovley et al., 1991; Lovley, 1993; Haveman and Pedersen, 2002). An injection of acetate as the electron donor not only stimulates  $Fe^{3+}$  reduction but also U(VI) reduction to U(IV), which has a low solubility and hence is less likely to be transported.

## **Control on Nitrate in Wetlands**

Nitrate is a major pollutant in surface and subsurface waters. Natural wetlands normally function as a sink for  $NO_3^-$ . Constructed and natural wetlands have been used

for  $\text{NO}_3^-$  removal from waters (Reilly et al., 2000; Lin et al., 2002; Rutherford and Nguyen, 2004). Both the retention time and the reduction capacity of the functioning zone of a wetland are important in the efficiency of  $\text{NO}_3^-$  removal. In general, a low redox condition favors  $\text{NO}_3^-$  removal with less  $\text{N}_2\text{O}$  being formed (Kralova et al., 1992), although an excess of electron donors under highly reducing conditions may favor dissimilatory reduction of  $\text{NO}_3^-$  to ammonium but not to  $\text{N}_2$  (King and Nedwell, 1985). When wetlands (e.g. riparian wetlands and rice fields) are intermittently or seasonally flooded, the redox conditions are variable and so is their ability for  $\text{NO}_3^-$  removal. Nitrate may not be efficiently removed by the functioning zone but be transported through, causing surface/subsurface water pollution. Such  $\text{NO}_3^-$  transport has been found in rice fields where high nitrogen fertilization rates are used (Zhu et al., 2000). Groundwater discharge contributes significantly to the  $\text{NO}_3^-$  load in rivers (Staver and Brinsfield, 1996; Dowling et al., 2004).

Organic carbon sources as electron donors are important in  $\text{NO}_3^-$  transformation in wetland soils (Vanoostrom and Russell, 1994; Weisner et al., 1994; Ingersoll and Baker, 1998; Hill and Cardaci, 2004). Microbial  $\text{Fe}^{3+}$  reduction contributes substantially to anaerobic carbon oxidation in freshwater soils and sediments (Roden and Wetzel, 1996; Yao et al., 1999) and should affect the pool of electron donors. Nevertheless, little information is available on the significance of microbial  $\text{Fe}^{3+}$  reduction in anaerobic carbon oxidation, and the role of  $\text{Fe}^{3+}$  reduction in  $\text{NO}_3^-$  transformation in wetland environments. The ability of the functioning zone to transform  $\text{NO}_3^-$  is of key importance

in determining how much  $\text{NO}_3^-$  may be transported through the functioning zone. Under carbon-limiting conditions, the availability of  $\text{Fe}^{3+}$ -centered intermediate AEAs, typically including  $\text{Mn}^{4+}$ , becomes important. Ferric iron-centered intermediate AEAs may be reduced, increasing the limitation of electron donors at certain depths before  $\text{NO}_3^-$  is transported there. The reduced iron compounds may promote chemoautotrophic  $\text{NO}_3^-$  reduction (Murase and Kimura 1997; Benz et al., 1998) but it is not expected to be as efficient as heterotrophic  $\text{NO}_3^-$  reduction based on thermodynamic principles. As a result, when  $\text{Fe}^{3+}$ -centered AEAs are abundant,  $\text{NO}_3^-$  transformation is likely lowered while  $\text{N}_2\text{O}$  as the end product relative to  $\text{N}_2$  increases and  $\text{NO}_3^-$  transport is promoted. Nitrous oxide is an important biogenic greenhouse gas with a global warming potential 310 times that of  $\text{CO}_2$  on a molecular basis (IPCC, 2001). Nitrous oxide emission at high rates is observed in treatment wetlands (Freeman et al., 1997; Fey et al., 1999; Mander et al., 2003). Evaluation of loading capacities of treatment wetlands for  $\text{NO}_3^-$  removal is needed to ensure effective  $\text{NO}_3^-$  removal while minimizing  $\text{N}_2\text{O}$  emission.

### **Control on Methane in Wetlands**

Methane is a major biogenic greenhouse gas, second only to  $\text{CO}_2$  in its contribution to global warming (IPCC, 2001). Wetlands are a major source of  $\text{CH}_4$  (IPCC, 2001). It is not reasonable to drain natural wetlands for  $\text{CH}_4$  mitigation due to their important economic and ecoenvironmental roles (Patrick, 1994), including their functioning as an active carbon and nitrate sink (Brix et al., 2001; Vitt et al., 2001; Whiting and Chanton, et al., 2001). Artificial wetlands are generally not an effective carbon sink but are an



important CH<sub>4</sub> source (IPCC, 2001). Many approaches have been tested for CH<sub>4</sub> mitigation in rice fields, including selective use of rice cultivars, timing and rate of organic and inorganic fertilizer applications, and, intermittent flooding through water management, etc (Sigren et al., 1997; Yagi et al., 1997; Wang et al., 2000; Wassmann et al. 2000; Krüger et al. 2001). Among these approaches, intermittent flooding is most effective and promising (Yagi et al., 1997).

The availability of reducible iron is an important soil characteristic in determination of CH<sub>4</sub> emission in rice soils (Wang et al., 1993b; Watanabe and Kimura 1999; Yao et al., 1999; Liu et al., 2003). Ferric iron can directly inhibit CH<sub>4</sub> production (van Bodegom et al., 2004). An increase in microbial Fe<sup>3+</sup> reduction, e.g. through Fe<sup>3+</sup> amendment, reduces CH<sub>4</sub> emission in continuously flooded rice soils (Jäkel and Schnell, 2000; Furukawa and Inubushi, 2002).

Rapid Fe<sup>2+</sup> reoxidation is found at the oxygenated soil-water interface and also in the oxidized rhizosphere where it is important in controlling root exudation and CH<sub>4</sub> dynamics (Frenzel et al., 1999; King & Garey 1999; Ratering & Schnell 2000). Ferric iron regeneration during non-flooding periods contributes to CH<sub>4</sub> mitigation upon reflooding (Sigren et al., 1997; Ratering and Conrad, 1998), and hence is important in determination of non-flooding durations required for effective CH<sub>4</sub> mitigation without the risks of reducing rice yield and increasing the emission of N<sub>2</sub>O (Henckel and Conrad, 1998; Cai et al., 1999).

In general, the importance of redox cycling of iron in regulation of CH<sub>4</sub> dynamics is

poorly understood. The effect of  $\text{Fe}^{3+}$  reduction on  $\text{CH}_4$  emission from rice paddies is only preliminarily considered in very recent model studies predicting  $\text{CH}_4$  emission from rice paddies (Matthews et al., 2000; van Bodegom et al., 2000). Large uncertainties still remain in evaluation of the impact of iron redox cycling on  $\text{CH}_4$  emission from rice paddies. Specifically, the roles of reduction and regeneration of  $\text{Fe}^{3+}$  need to be evaluated in water management based options for  $\text{CH}_4$  mitigation in rice fields.

### **Bioelectricity Generation from Anaerobic Environments for Pollution Control**

Large amounts of potential energy are associated with organic matter in anaerobic soils/sediments but are difficult to utilize. When a soil/sediment develops highly reducing conditions, bioelectrochemically active microorganisms such as iron-reducing bacteria can use an electrode (e.g. graphite plate) as the electron acceptor when it is electrically connected to another electrode placed in the oxidized overlying water to support bioelectricity generation (Bond et al., 2002; Reimers et al., 2001). The addition of an external electron shuttling substance is not necessary in this process, providing a possibility to directly harvest power from anaerobic sediments/soils.

The microbially harvested power normally ranges from 10 to 100  $\text{mW m}^{-2}$  (Liu et al., 2004) and can be used to drive low-power sensors in marine environments (Bond et al., 2002; Reimers et al., 2001). Difficulties remain in collecting, concentrating, and storage of this energy for practical applications. Nevertheless, since the internal resistance of a microbial fuel cell is relatively high, low bioelectricity yield is not necessarily as important a goal as using this energy for in situ bioelectrochemical pollutant removal.

During bioelectricity generation, waste organic matter (e.g. from wastewater) is bioelectrochemically oxidized by  $O_2$  (Liu et al., 2004) to prevent or lower the formation of  $CH_4$  (Rabaey et al., 2003) and oxidized pollutants such as  $NO_3^-$  can be bioelectrochemically reduced when or where  $O_2$  is not available (Gregory et al., 2004). Nitrate as a major pollutant in drinking water can be removed with physico/chemical and biological processes (Kapoor and Viraraghavan, 1997). With the microbial fuel cell approach, organic carbon from wastewaters, soils, and sediments is available as the reductant for  $NO_3^-$  removal even with no direct contact with the water. The use of microbially mediated reductants as energy sources by the biobattery approach also makes it different from electrochemical/bioelectrochemical  $NO_3^-$  removal by an electric current field (Feleke and Sakakibara, 2001; Zaveri and Flora, 2002; Beschkov et al., 2004).

### **Research Objectives**

Wetlands are a major source of  $CH_4$  and a sink for  $NO_3^-$ . Artificial wetlands can also be a source of  $NO_3^-$ . Iron redox processes play important roles in  $NO_3^-$  and  $CH_4$  biogeochemistry in wetland environments. These roles are poorly quantified due to the heterogeneity of iron redox processes. The temporal and spatial distribution of iron-centered intermediate redox processes, typically including manganese redox processes, not only controls methanogenesis heterogeneity but also affects the fate of  $NO_3^-$  in wetland soils.

In this dissertation, soil suspension, soil column, and pot experiments were carried out to investigate the influences of iron-centered intermediate redox processes on  $NO_3^-$

reduction and CH<sub>4</sub> formation in rice soils. The importance of microbial Fe<sup>3+</sup> reduction in timing and scaling of denitrification and methanogenesis was evaluated in anoxic soil suspensions (Chapter 2). The role of cycling of Fe<sup>3+</sup>-centered AEAs in temporal and vertical variations of N<sub>2</sub>O and CH<sub>4</sub> production were evaluated in flooded soil columns (Chapter 3). The effect of a pool of Fe<sup>3+</sup>-centered intermediate AEAs on NO<sub>3</sub><sup>-</sup> dynamics was evaluated in flooded soil columns at different percolation rates (Chapter 4). Microbial activity responsible for the reduction of Fe<sup>3+</sup>-centered AEAs was estimated using an iron redox processes-dominated intermediately reducing zone with comparison to other redox zones (Chapter 5). The ability of a flooded rice soil as a reductant source to bioelectrochemically remove NO<sub>3</sub><sup>-</sup> from water was evaluated (Chapter 6). A pot experiment was carried out to study effects of Fe<sup>3+</sup> reduction and regeneration on NO<sub>3</sub><sup>-</sup> percolation and emissions of N<sub>2</sub>O and CH<sub>4</sub> from a soil-rice system (Chapter 7). Chapter 8 was a summary of the results obtained from the above six experiments.

## **CHAPTER 2**

### **EFFECT OF MICROBIAL IRON REDUCTION ON DENITRIFICATION AND METHANOGENESIS IN ANOXIC SOIL SLURRIES**

#### **Introduction**

Anaerobic carbon oxidation occurs after the depletion of oxygen in the porewater in flooded soils, coupled to sequential reduction of a series of electron acceptors, mainly  $\text{NO}_3^-$ ,  $\text{Mn}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$  and finally  $\text{CO}_2$  (Patrick and DeLaune, 1972; Reddy et al., 1989). In freshwater soils, the presence of the element S is normally low while Fe is an abundant element. While most iron compounds are particulate and largely incapable of being reduced by microbial respiration, due to the large amount and variety of forms of iron present, microbial  $\text{Fe}^{3+}$  reduction is an electron accepting process dominant or second only to methanogenesis in anaerobic carbon decomposition in most freshwater soils (Yao et al., 1999).

A number of factors affect microbial  $\text{Fe}^{3+}$  reduction in soil. Acidic conditions increase solubility of iron compounds and may increase microbial  $\text{Fe}^{3+}$  reduction if such pH conditions are physiologically acceptable. The presence of quinone-containing substances such as humic substances may facilitate microbial reduction of particulate  $\text{Fe}^{3+}$  (Straub et al., 2001; Luu and Ramsay, 2003). More importantly,  $\text{Fe}^{3+}$  is capable of regeneration. Iron redox cycling occurs so intensively around aerobic/anaerobic interfaces in wetland and aquatic environments that microbial  $\text{Fe}^{3+}$  reduction even contributes substantially to anaerobic carbon mineralization in marine sediments (Aller et al., 1996; VanCappellen and

Wang, 1996). Because of its importance in anaerobic carbon decomposition, microbial  $\text{Fe}^{3+}$  reduction plays many important ecoenvironmental roles in organic pollutant degradation, toxic metal mobilization and immobilization in soils and sediments,  $\text{NO}_3^-$  dynamics, and  $\text{CH}_4$  biogeochemistry in wetland and aquatic environments (Lovley, 1991; Lovley et al., 1994; Roden and Wetzel, 1996; Yao et al., 1999; Senn et al., 2002).

In wetland soils, microbial  $\text{Fe}^{3+}$  reduction as an intermediate redox process is an important factor determining the duration of intermediate reducing conditions between the depletion of nitrate and initiation of methanogenesis, a condition where  $\text{N}_2\text{O}$  and  $\text{CH}_4$  fluxes are low (Yu and Patrick, 2003). As such,  $\text{Fe}^{3+}$  reduction is an important factor affecting  $\text{CH}_4$  emissions from rice fields (Wang et al., 1993b; Watanabe and Kimura 1999; 1999; Liu et al., 2003). Rice fields are a major biogenic source of  $\text{CH}_4$  (IPCC, 2001), which is second only to  $\text{CO}_2$  in its contribution to global warming.

Ferric iron reduction may directly and indirectly affect methanogenesis and denitrification in anoxic conditions. In homogenized soil slurries,  $\text{Fe}^{3+}$  reduction suppresses methanogenesis (Achthich et al., 1995) but may con occur with denitrification especially when available carbon is abundant. While  $\text{Fe}^{3+}$  reduction con occurring with denitrification may be difficult to detect due to rapid chemical and/or biological reoxidation of  $\text{Fe}^{2+}$  that is produced (Finneran et al., 2002), this process may promote  $\text{N}_2\text{O}$  formation (Brons et al., 1991) and even affect overall denitrification. In field conditions, due to soil heterogeneity, microbial  $\text{Fe}^{3+}$  reduction may con occur with denitrification and methanogenesis on various spatial scales and more intensively affect them through

redistribution and regeneration of  $\text{Fe}^{3+}$  in the soil (Benz et al., 1998; Nielsen et al., 1998; Frenzel et al., 1999; Rysgaard et al., 2001).

In this chapter, we selected two rice soils differing in pH and iron content to study the effects of microbial  $\text{Fe}^{3+}$  reduction on denitrification and methanogenesis in homogenized soil slurries. Studies on the effects of microbial  $\text{Fe}^{3+}$  reduction on denitrification and methanogenesis under more natural soil conditions were reported in other chapters.

## Materials and Methods

### Soils

Two rice soils were used in the experiments. One is a Crowley silt loam soil (fine, smectitic, hyperthermic Typic Albaqualfs) from Crowley, Louisiana (LA soil) and the other is a Beaumont clay soil (fine, montmorillonitic, thermic Entic Pelluderts) from Beaumont, Texas (TX soil). The soil samples were collected from the top layer (0-20 cm), air-dried, stored at room temperature (20 °C), and sieved (<1-mm) for the slurry experiment. Soil characteristics of interest were analyzed and shown in Table 2.1.

**Table 2.1** Selected characteristics of the soils.

Soil	pH	OM (%)	Total N (%)	P	K	Extractable Fe †	Extractable Mn †	Extractable S ‡
mg kg <sup>-1</sup>								
LA	7.3	1.67	0.07	42	291	68.2	19.5	10.7
TX	5.1	2.54	0.11	68	104	115.6	35.1	38.4

† Extracted with DTPA (diethylene triamine pentaacetic acid) solution to remove soluble and labile solid phases (Lindsay and Norvell, 1978).

‡ Extracted with ammonium acetate and acetic acid solution to remove S that was presumed to be mostly  $\text{SO}_4^{2-}$  (Bardsley and Lancaster, 1960).

## Slurry Experiment

The microcosm technique of Patrick et al. (1973) was used in the slurry experiment with some modification (Yu and Patrick, 2003). The soil slurry was prepared by adding 400 g air-dried soil and 1600 ml deionized water into a 2300-ml Erlenmeyer flask, with or without ferrihydrite amendment at  $40 \mu\text{mol Fe}^{3+} \text{ g}^{-1}$ . Ferrihydrite was prepared (Jäckel and Schnell, 2000) and resuspended in deionized water at pH 7. The soil slurry was aerobically preincubated under constant stirring at  $25^\circ\text{C}$  for 4 days. Then 4 g of ground rice straw was added as an energy source and potassium nitrate was added at  $8 \mu\text{mol N g}^{-1}$  into the soil slurry. The flask was capped with a rubber stopper in which a septum was installed for gas sampling, and a gas inlet and outlet were installed for headspace purging. The headspace was purged with pure  $\text{N}_2$  for 20 min to start the anaerobic incubation. Pure  $\text{N}_2$  was bubbled through the suspension at a rate of  $15 \text{ ml min}^{-1}$  to maintain anaerobic conditions during the incubation. Instead of using treatment replication, an approach of frequent sampling and measurement was used to ensure the duplicability and accuracy of experiment with this homogenized microcosm containing a large soil mass (Yu and Patrick, 2003).

At hours 0, 12, 24, 48, 72, 120 and 172 after the start of the anaerobic incubation, 10 ml of soil slurry was anaerobically taken from the flask, centrifuged, filtered ( $<0.45 \mu\text{m}$ ) and stabilized 10 ml of  $\text{NO}_3^-$  buffer (APHA, 1995) for  $\text{NO}_3^-$  determination with an ion meter coupled to a combination  $\text{NO}_3^-$  selective electrode (detection limit  $7 \times 10^{-6} \text{ M}$ ). Ten ml of deionized water was added after the sampling of soil slurry to compensate for the volume change of soil slurry. The removal of soil was considered in the calculation of the



rates of  $\text{NO}_3^-$  reduction and production of  $\text{N}_2\text{O}$ ,  $\text{CO}_2$ , and  $\text{CH}_4$  on a mass basis. Redox potential and gas production were normally daily measured until  $\text{CH}_4$  production from the microcosm became less variable, and pH was measured on selected dates. The redox potential was measured before gas sampling and was reported as the mean reading (Eh) of Pt electrodes (n=2) coupled to a saturated calomel reference electrode after correction to the standard  $\text{H}_2$  electrode by adding 245 mV to the instrument reading. The microcosm was purged with  $\text{N}_2$  at a higher flow rate (at least double than normal) for two hours before gas sampling. Then gas samples were taken three times from the headspace of the microcosm at 0, 20, and 40 minutes after stop purging and analyzed for  $\text{N}_2\text{O}$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  concentrations (see below). Ten ml of soil slurry (n=2) was taken at the end of anaerobic incubation for measurement of the content of microbially reducible iron, which was extracted by 0.5 M HCl (Lovley and Phillips, 1986) and determined by ICP.

### **Analysis of Gas Samples**

Gas samples were taken and analyzed with a Tremetrics 9001 gas chromatograph (GC) using an electron capture detector (ECD) for determining  $\text{N}_2\text{O}$  and a flame ionization detector (FID) for determining concentrations of  $\text{CH}_4$  and  $\text{CO}_2$  after calibration with certified standards. A methanizer catalyst option was installed after the GC column to convert  $\text{CO}_2$  to  $\text{CH}_4$  for FID detection.

### **Calculation and Statistical Analysis**

Gas production rate was calculated by linear regression of the three analyses in 40 minutes (Yu and Patrick, 2003). The dissolution of  $\text{N}_2\text{O}$  ( $5.07 \times 10^{-4}$ , mole ratio),  $\text{CO}_2$

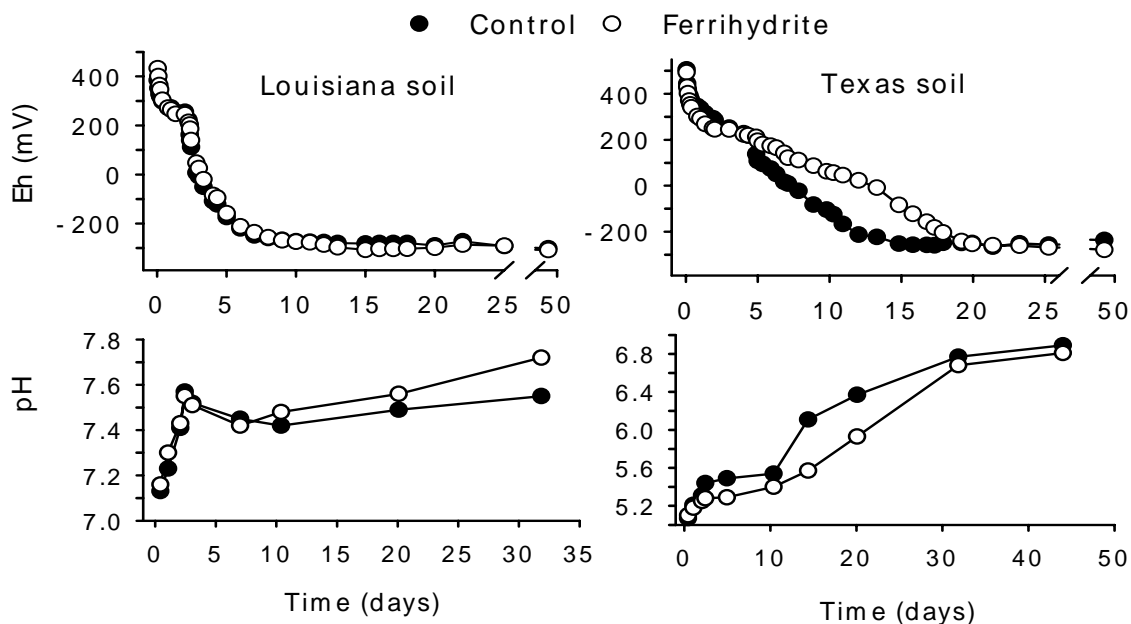
(0.707), and CH<sub>4</sub> (0.028) (Handbook of Chemistry and Physics, 1991) in the liquid phase was considered in their production rate calculation. The cumulative production of N<sub>2</sub>O or CH<sub>4</sub> in the slurry was the sum of the production rate on each sampling date multiplied by the time interval, which was calculated from the midpoints of neighboring sampling dates. The cumulative production of CO<sub>2</sub> was not calculated due to the difficulty in considering the effect of pH on CO<sub>2</sub> solubility in the microcosm.

Statistical analysis was conducted with SAS (SAS Institute Inc., 1999-2001). The curves of NO<sub>3</sub><sup>-</sup> reduction and N<sub>2</sub>O production in the soil slurries versus time or Eh were linearly or exponentially fitted. The statistical level of significance was chosen at  $\alpha = 0.05$  for all statistical analysis.

## **Results**

### **Temporal Change of Redox Potential and pH**

The Eh decrease was more rapid in the Louisiana soil slurries than in the Texas soil slurries (Fig. 2.1). Ferrihydrite amendment had little effect on Eh decrease in the Louisiana soil, but apparently delayed the Eh decrease in the Texas soil between days 5 and 18. The pH increase during the anaerobic incubation was less than 0.6 pH unit in the Louisiana soil, but close to 2 units in the Texas soil (Fig. 2.1). Ferrihydrite amendment slightly increased the pH in the Louisiana soil after day 10, but apparently delayed the pH rise in the Texas soil between days 5 and 20.



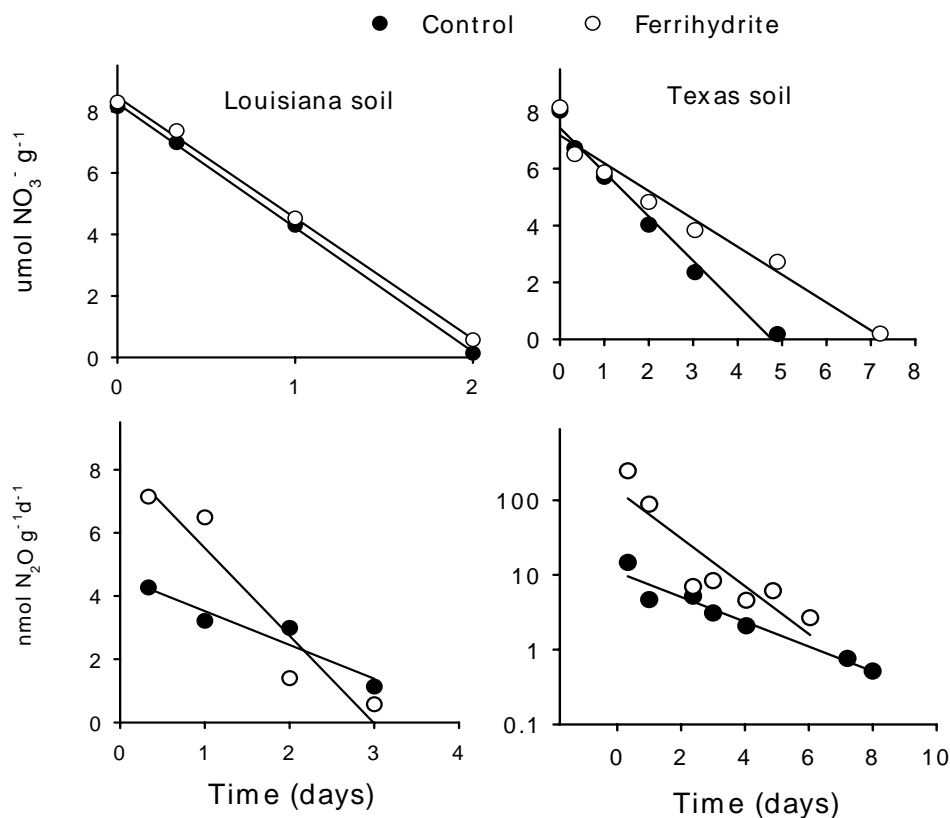
**Fig. 2.1** Temporal changes in Eh and pH in the anoxic soil slurries.

### Nitrate Reduction and Nitrous Oxide Production

Nitrate reduction was rapid in the Louisiana soil at a linear rate of  $4.01 \mu\text{mol N g}^{-1} \text{d}^{-1}$  ( $p < 0.001$ ) for 48 h and was little affected by ferrihydrite amendment (Fig. 2.2).

Ferrihydrite amendment in the Texas soil lowered the  $\text{NO}_3^-$  reduction rate by 37% from  $1.56$  to  $0.98 \mu\text{mol g}^{-1} \text{d}^{-1}$ , and prolonged the depletion time by 48% from 117 to 172 h.

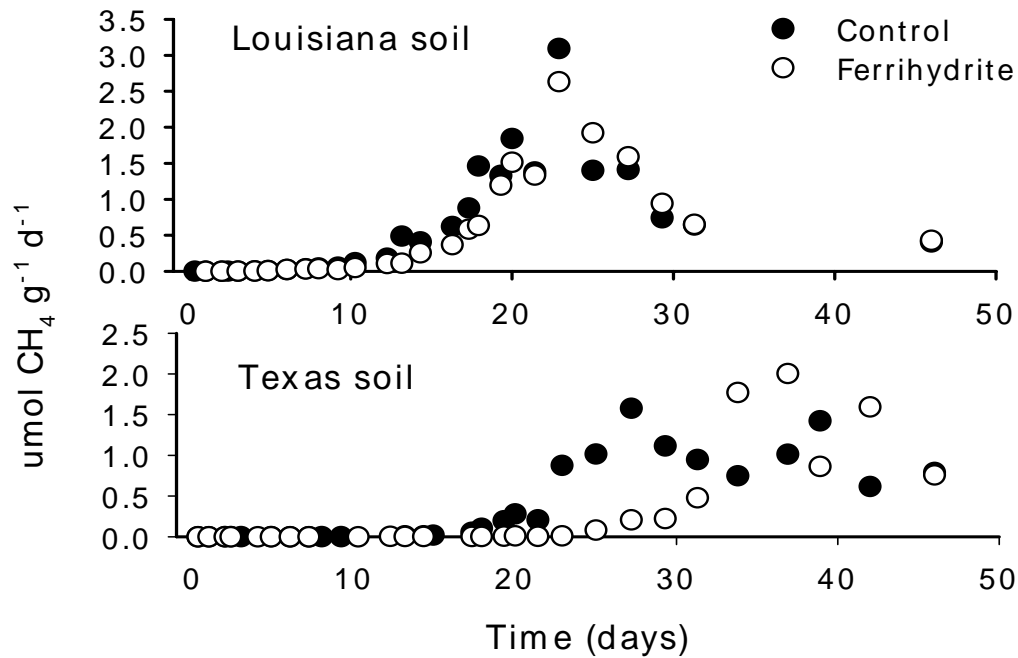
Nitrous oxide production linearly decreased with time in the Louisiana soil ( $p < 0.05$ ), but exponentially decreased in the Texas soil ( $p < 0.01$ ) (Fig. 2.2). Ferrihydrite amendment dramatically increased  $\text{N}_2\text{O}$  production at beginning of the incubation in both soils.



**Fig. 2.2**  $\text{NO}_3^-$  reduction and  $\text{N}_2\text{O}$  production in the soil slurries.

### Methane Production

Methanogenesis was more rapidly initiated in the Louisiana soil than in the Texas soil (Fig. 2.3). Ferrihydrite amendment delayed the initiation of significant  $\text{CH}_4$  production ( $>10 \text{ nmol g}^{-1} \text{ d}^{-1}$ ) from day 5 to 6 in the Louisiana soil, and from day 13 to 20 in the Texas soil. Ferrihydrite amendment lowered the peak of  $\text{CH}_4$  production by 15% in the Louisiana soil, but increased production by 27% in the Texas soil. The reductions in cumulative  $\text{CH}_4$  production by ferrihydrite amendment were 23% in the Louisiana soil and 92% in the Texas soil when the  $\text{CH}_4$  production peaks in the control treatments were observed (on days 23 and 27, respectively) (Table 2.2). The overall  $\text{CH}_4$  reductions were 11% in the Louisiana soil and 13% in the Texas soil during the 46-day incubation.



**Fig. 2.3** CH<sub>4</sub> production in the soil slurries.

**Table 2.2** Effects of ferrihydrite amendment on ferric iron reduction, nitrate reduction, and production of N<sub>2</sub>O and CH<sub>4</sub> in the anaerobically incubated soil slurries.

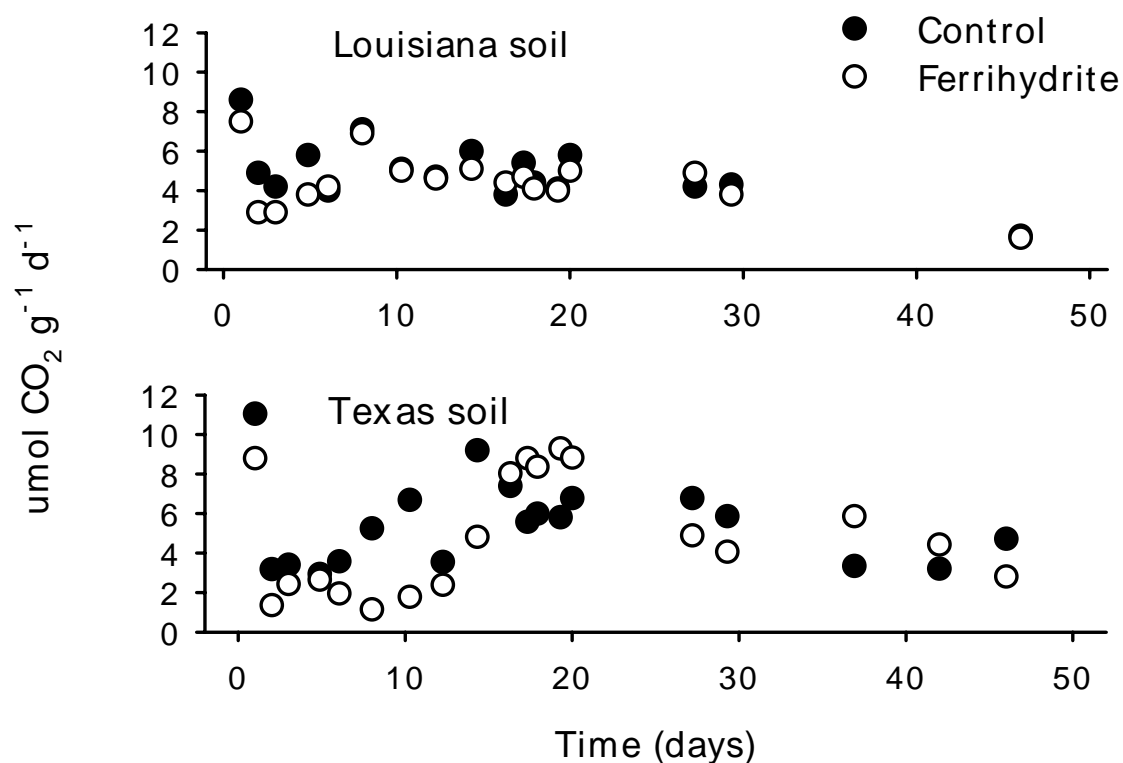
Soil	Fe <sup>3+</sup> amended (μmol g <sup>-1</sup> )	Fe <sup>3+</sup> reduced (μmol g <sup>-1</sup> )	Nitrate reduction (μmol g <sup>-1</sup> d <sup>-1</sup> )	N <sub>2</sub> O production (nmol g <sup>-1</sup> d <sup>-1</sup> )	CH <sub>4</sub> formation 1 (μmol g <sup>-1</sup> d <sup>-1</sup> ) †	CH <sub>4</sub> formation 2 (μmol g <sup>-1</sup> d <sup>-1</sup> ) ‡
LA	0	63.3	4.01	2.8	0.66	0.85
LA	40	97.0	3.86	4.4	0.51	0.76
TX	0	196.8	1.56	2.5	0.29	0.54
TX	40	214.6	0.98	39.0	0.024	0.47

† When the corresponding control treatment reached the maximum methane production rate (on days 23 and 27, respectively).

‡ The whole incubation period (day 46).

## Carbon Dioxide Production

There was a decrease and then an increase of CO<sub>2</sub> production in the Louisiana soil slurries in the first 10 days, and in the Texas soil slurries in the first 20 days (Fig. 2.4). Thereafter, CO<sub>2</sub> production in all soil slurries was less variable or in a trend of decreasing at low rates along the time course. Ferrihydrite amendment decreased CO<sub>2</sub> production in the Louisiana soil in the first 5 days and in the Texas soil in the first 10 days.



**Fig. 2.4** CO<sub>2</sub> production in the soil slurries.

## Nitrous Oxide and Methane Production within Different Redox Ranges

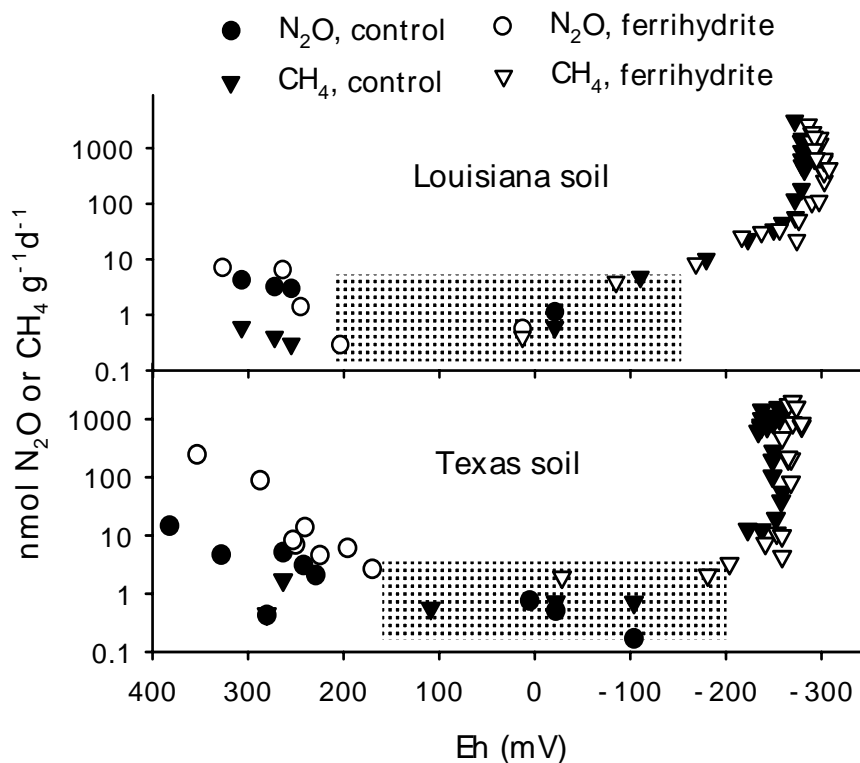
Significant N<sub>2</sub>O production (> 2 nmol g<sup>-1</sup> d<sup>-1</sup>) was found in the weakly reducing range (>200 mV), with the rate decreasing linearly with a decrease of Eh in the Louisiana

soil slurries ( $p < 0.05$ ), and decreasing exponentially in the Texas soil slurries (Fig. 2.5)

( $p < 0.01$ ). Significant  $\text{CH}_4$  production ( $> 10 \text{ nmol g}^{-1} \text{ d}^{-1}$ ) was found in the highly

reducing range ( $< -200 \text{ mV}$ ), with the rate increasing exponentially with a decrease of Eh

in all soil slurries ( $p < 0.01$ ).



**Fig. 2.5**  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production in different Eh ranges in the soil slurries. In the shaded Eh ranges  $\text{N}_2\text{O}$  and  $\text{CH}_4$  are produced at low rates.

The specific Eh range within which  $\text{N}_2\text{O}$  and  $\text{CH}_4$  were both produced at low rates ( $< 2$  and  $10 \text{ nmol g}^{-1} \text{ d}^{-1}$ , respectively) was 200 to  $-150 \text{ mV}$  in the Louisiana soil, and 150 to  $-200 \text{ mV}$  in the Texas soil. Iron amendment had little effect within this Eh range, but increased the production of  $\text{N}_2\text{O}$  in the weakly reducing range, and reduced the production of  $\text{CH}_4$  in the highly reducing range of both soils.

## Ferric Iron Reduction

The content of indigenous reducible iron was lower but ferrihydrite amendment induced more  $\text{Fe}^{3+}$  reduction in the Louisiana soil than in the Texas soil (Table 2.2). Without considering the contribution of microbial reduction of  $\text{Mn}^{4+}$ ,  $\text{Fe}^{3+}$  reduction as an electron accepting process was second only to methanogenesis in the Louisiana soil but dominant in the Texas soil during the 46-day incubation (Table 2.3).

**Table 2.3** Electron transfer in major redox processes of the soil slurries.

Soil	Ferrihydrite amendment ( $\mu\text{mol g}^{-1}$ )	Electron transfer ( $\mu\text{mol g}^{-1}$ )			
		$\text{NO}_3^-$ reduction †	$\text{Fe}^{3+}$ reduction ‡	$\text{SO}_4^{2-}$ reduction §	$\text{CH}_4$ production ¶
LA	0	40.0	63.3	2.7	155.7
	40	40.0	97.0	2.7	140.4
TX	0	40.0	196.8	9.6	98.7
	40	40.0	214.6	9.6	86.6

† Assuming background  $\text{NO}_3^-$  content and  $\text{NO}_3^-$  transformation other than denitrification to  $\text{N}_2$  were negligible.

‡ One electron was required for the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ .

§ Assuming extractable S was in  $\text{SO}_4^{2-}$  form and reduced to sulfide.

¶ Assuming organic carbon at oxidation state zero was the only electron donor converted to either  $\text{CO}_2$  or  $\text{CH}_4$  (Yao et al., 1999).

## Discussion

Three major redox processes, denitrification,  $\text{Fe}^{3+}$  reduction and methanogenesis, in the two anaerobically incubated soils were measured. Sulfate reduction was not quantified but was presumably less important as a redox process than  $\text{Fe}^{3+}$  reduction (Table 2.3). Microbial  $\text{Mn}^{4+}$  reduction was also expected to be of minor importance compared to  $\text{Fe}^{3+}$  reduction (Yao et al., 1999), especially considering the relatively low



content of extractable Mn in each soil. Microbial  $\text{Fe}^{3+}$  reduction as a major alternative electron accepting process, together with soil pH, was found to be important in the succession of redox processes and timing and scaling of  $\text{N}_2\text{O}$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  production in both soils.

A higher content of indigenous reducible  $\text{Fe}^{3+}$ , but less favorable pH conditions for anaerobes were probably the two major factors resulting in the slower development of the full range of redox processes in the Texas soil compared to the Louisiana soil, as indicated by the differences in Eh,  $\text{NO}_3^-$  reduction, and  $\text{N}_2\text{O}$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  production. Acidic conditions retard denitrification activity, especially  $\text{N}_2\text{O}$  reduction (Burford and Bremner, 1975) and not are favorable to methanogenesis (Wang et al., 1993a). The pH values in the Texas soil slurries were not more than 6.1 when denitrification and substantial  $\text{CH}_4$  production were initiated; the pH varied much less in a range more favorable to denitrifiers and methanogens in the Louisiana soil slurries. As a result, lower  $\text{NO}_3^-$  reduction with more  $\text{N}_2\text{O}$  production and later initiation of methanogenesis at lower rates occurred in the Texas soil compared to the Louisiana soil.

Nevertheless, besides the less favorable pH conditions, a higher level of reducible iron also contributed to the lower  $\text{NO}_3^-$  reduction with higher  $\text{N}_2\text{O}$  production in the Texas soil. This effect was evidenced by ferrihydrite amendment in each soil. Denitrification did not completely inhibit the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , especially in the presence of sufficient carbon source (Acht nich et al., 1995). The formed  $\text{Fe}^{2+}$  may rapidly react with nitrite, an intermediate product of denitrification, to promote formation of NO

and  $\text{N}_2\text{O}$  (Brons et al., 1991). Thus, besides soil pH, cycling of Fe between oxidized and reduced states in the presence of  $\text{NO}_3^-$  also affected gaseous N formation, including  $\text{N}_2\text{O}$ . Ferrihydrite amendment promoted  $\text{Fe}^{2+}$  formation, resulting in higher  $\text{N}_2\text{O}$  production in both soils. This effect was augmented by a low soil pH to further decrease the overall  $\text{NO}_3^-$  reduction in the Texas soil.

Similarly, besides the less favorable pH conditions, higher levels of reducible  $\text{Fe}^{3+}$  also contributed to the later initiation of methanogenesis in the Texas soil slurries compared to the Louisiana soil slurries as evidenced by the suppression of methanogenesis in each soil upon ferrihydrite amendment. In the Louisiana soil, pH, Eh and timing of methanogenesis was slightly affected by ferrihydrite amendment, but the rate of  $\text{CH}_4$  production was consistently reduced until day 23. Despite the rice straw amendment, increasing the availability of AEAs relative to available carbon sources by ferrihydrite amendment appeared to be important in reducing  $\text{CH}_4$  production in the Louisiana soil. In contrast, ferrihydrite amendment in the Texas soil apparently delayed  $\text{NO}_3^-$  reduction and change of pH and Eh, resulting in a greater delay of methanogenesis in the Texas soil. Nevertheless, ferrihydrite amendment in the Texas soil changed the soil pH from 6.6 to 6.8 when methanogenesis peaked, a significant favorable pH change for methanogens (Wang et al., 1993a), resulting in the higher  $\text{CH}_4$  peak.

The temporal changes in  $\text{CO}_2$  production in the period during and soon after denitrification were mainly attributed to the need of initiation of fermenting activity for providing electron donors for the redox processes other than denitrification. Fermenting

bacteria were less competitive than denitrifiers (Dassonville et al., 2004). Nevertheless, according to the rate of CO<sub>2</sub> produced to that of NO<sub>3</sub><sup>-</sup> reduced, fermenting activity was not completely suppressed by denitrification activity but was weakened to some extent by ferrihydrite amendment as evidenced by generally lowered CO<sub>2</sub> production during the denitrifying stage.

Despite the observed effects of Fe<sup>3+</sup> reduction on the timing and rates of denitrification and methanogenesis, the interactions of Fe<sup>3+</sup> reduction with denitrification and methanogenesis appeared to be underestimated in the homogenized soil slurries. Iron amendment could extend the duration of an intermediate Eh but did not change the specific Eh range (e.g., +150 to -150 mV) within which N<sub>2</sub>O and CH<sub>4</sub> are produced at low rates (Yu and Patrick, 2003). This Eh range was largely occupied by iron-centered intermediate redox processes, implying that overlapping of Fe<sup>3+</sup> reduction with denitrification and methanogenesis was small in the unidirectional succession of redox processes in the homogenized soil slurries. However, such overlapping in heterogeneous field conditions is observed through repeated recycling of Fe<sup>3+</sup> (Frenzel et al., 1999; King and Garey, 1999; Ratering and Schnell, 2000). The effects of recycling of Fe<sup>3+</sup>-centered AEAs on NO<sub>3</sub><sup>-</sup> reduction and methanogenesis in rice soils will be investigated under more realistic conditions in later chapters.

## **Conclusions**

Microbial Fe<sup>3+</sup> reduction as a major alternative electron accepting process affected denitrification and methanogenesis in anoxic soil slurries. An increase in reducible Fe<sup>3+</sup>

content by ferrihydrite amendment changed environmental conditions, specifically pH, Eh and AEA availability, to stimulate N<sub>2</sub>O production and suppress CH<sub>4</sub> production. The increase in N<sub>2</sub>O flux by ferrihydrite amendment was significant but temporary while the suppression of CH<sub>4</sub> production was extended for considerable periods. Ferrihydrite amendment had little effect on the Eh range within which both N<sub>2</sub>O and CH<sub>4</sub> were produced at low rates.

### **CHAPTER 3**

## **TEMPORAL AND VERTICAL VARIATIONS OF NITROUS OXIDE AND METHANE PRODUCTION AFFECTED BY IRON-CENTERED INTERMEDIATE REDOX PROCESSES IN A FLOODED SOIL**

### **Introduction**

Most soils undergo a change from oxic to anoxic conditions upon flooding. Oxygen is depleted soon except at and above the aerobic/anaerobic interface while the reduction of  $\text{NO}_3^-$ ,  $\text{Mn}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$ , and  $\text{CO}_2$  occurs sequentially (Peters and Conrad, 1996) in the anaerobic layer. The sequential redox processes under field conditions normally overlap and interact to some extent, resulting in heterogeneous redox conditions in wetland soils (Gao et al., 2002).

Iron and manganese redox processes are two major intermediate redox processes in soils and sediments, and in general, iron redox chemistry is of central importance considering the abundance and the ability of redox cycling of iron relative to manganese (Horne, 1978; Stumm and Morgan, 1981). Iron-centered intermediate redox processes closely interact with many important processes such as  $\text{NO}_3^-$  reduction, metal mobilization/immobilization and methanogenesis in wetland and aquatic environments (Ratering and Conrad, 1998; Ratering and Schnell, 2001; Weber et al., 2001; Senn and Hemond, 2002). Nevertheless, in general these interactions have not been well established under field conditions due to the heterogeneous occurrence of cycling of  $\text{Fe}^{3+}$ -centered AEAs. In laboratory slurry studies, the succession of redox processes is normally unidirectional, from the oxic condition to the weakly reducing through

intermediately reducing to highly reducing conditions, and the multidirectional interactions are likely underestimated or obscured.

While cycling of  $\text{Fe}^{3+}$ -centered AEAs normally occurs around aerobic/anaerobic interfaces of wetland and aquatic environments, largely mediated by  $\text{O}_2$  diffusion (Frenzel et al., 1999; Rysgaard et al., 2001), this thin redox zone may be expanded under some conditions. For example, low levels of soil organic carbon, the abundant presence of one or more types of AEAs (e.g.  $\text{NO}_3^-$  and  $\text{Fe}^{3+}$ ), and low temperatures may all promote the expansion of iron redox cycling-centered intermediate redox zones in subsurface and surface anaerobic environments (Ludvigsen et al., 1998).

Specifically, since  $\text{NO}_3^-$  as an oxidant is soluble and readily reducible,  $\text{NO}_3^-$  diffusion is able to modify the redox profile to a greater extent than  $\text{O}_2$  diffusion through stimulating heterotrophic denitrification and recycling of AEAs such as  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  (Klüber and Conrad, 1998). Consequently, iron-centered intermediate redox processes may be vertically expanded and temporally extended in the soil and their interactions with other processes may be studied.

Methanogenesis and denitrification are major redox processes in freshwater soils and sediments. Cycling of  $\text{Fe}^{3+}$ -centered intermediate AEAs around aerobic/anaerobic interfaces, together with organic carbon inputs from aquatic plants, largely controls temporal and spatial variations of methanogenesis in these environments. The influence of  $\text{Fe}^{3+}$ -centered AEAs is important to  $\text{CH}_4$  flux in rice paddies and should be useful in estimating and predicting  $\text{CH}_4$  flux (Matthews et al., 2000; van Bodegom et al., 2000).

Cycling of  $\text{Fe}^{3+}$  affects  $\text{N}_2\text{O}$  formation from  $\text{NO}_3^-$  reduction even in soil slurries (Chapter 2). Such interaction may be more intensive under field conditions. In this study we used  $\text{NO}_3^-$  diffusion to create temporal and vertical variations in availability of electron donors and recycling of  $\text{Fe}^{3+}$ -centered intermediate AEAs in a rice soil. The importance of cycling of  $\text{Fe}^{3+}$ -centered intermediate AEAs in temporal and vertical variations of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production was evaluated.

## **Materials and Methods`**

### **Soil Column Incubation**

A rice soil, taken from a rice field at the Crowley Rice Experimental Station, Crowley, Louisiana, was used in the experiment. For the soil properties, see Table 2.1 in Chapter 2.

Plastic syringes (Monoject, 140 ml with catheter tip) were used for soil column incubations. The inner diameter and length of the syringe (not including the tip) were 3.7 and 15 cm, respectively. The syringe was loaded with 135 g soil (dry weight) and gently shaken to form a 10-cm soil column. Deionized water was added to the syringe to saturate the soil column. The soil column was continuously flooded with a 2-cm layer of overlying water. The syringe was incubated at 22°C in the dark. On day 17 of the incubation when the redox potential (Eh) profile in the soil column was relatively stable, the overlying water of the soil column was amended with 0 or 10.5 mg  $\text{NO}_3^-$ -N as potassium nitrate (equivalent to 0 or 35 mM  $\text{NO}_3^-$ ). The unamended and amended soil columns were continuously incubated as long as 120 days, and Eh and production of  $\text{N}_2\text{O}$ ,

CH<sub>4</sub>, and CO<sub>2</sub> with depth were measured at different times during the incubation.

### **Redox Profile before and after Nitrate Diffusion**

Five soil columns were used for the measurement of the Eh profile before and after the NO<sub>3</sub><sup>-</sup> amendment on day 17 until day 120 on various frequencies (daily to weekly). Platinum electrodes were installed along the sidewall of the syringe before incubation, corresponding to depths of 0.1, 0.5, 1.5, 2.5, 4, and 6 cm in the soil column. The electrodes were tested with quinhydrone-saturated standard pH solutions (Patrick et al., 1996). A calomel reference electrode was used during Eh measurement using a small tube containing a salt agar bridge immersed in the overlying water. The Eh was reported after correction against the standard H<sub>2</sub> electrode by adding 245 mV to the instrument reading.

### **Production of Nitrous Oxide, Methane and Carbon Dioxide**

On days 15, 30, and 60 (and also on day 120 for the NO<sub>3</sub><sup>-</sup>-amended soil column) of the incubation, one set (n=3) of the control or NO<sub>3</sub><sup>-</sup>-amended soil columns was sectioned for measurement of the production of N<sub>2</sub>O, CH<sub>4</sub>, and CO<sub>2</sub> in the soil column. The top part of the syringe above the soil was cut off in a glove bag under a N<sub>2</sub> atmosphere. The overlying water was discarded and the soil layers of 0-1, 1-2, 3-4, or 6-7 cm was extruded by pushing up the syringe plunger into a 125-ml flask containing 50 ml deionized water, and an anoxic soil slurry was maintained with a magnetic stirrer under a N<sub>2</sub> atmosphere. Five ml of the soil slurry was transferred with a plastic syringe into a test tube (16 x125 mm) to which had been added 1 ml of either a 0 or 12 mM acetate solution, and the tube was capped with a rubber stopper under a N<sub>2</sub> atmosphere. One set of the tubes with no



acetate addition was treated with an injection of 2 ml  $\text{H}_2$  per tube after taking 2 ml gas from the tube headspace. All the tubes were incubated at 22 °C under shaking (120 oscillations per min). Gas samples for the tubes without substrate addition were taken after a 2-h of incubation for determination of  $\text{N}_2\text{O}$  concentration. Gas samples from all tubes were taken after 11-12 h incubation for determination of  $\text{CH}_4$  and  $\text{CO}_2$  concentrations with a Tremetrics 9001 gas chromatograph (GC). The  $\text{N}_2\text{O}$ ,  $\text{CH}_4$ , and  $\text{CO}_2$  production rates were expressed as mass cumulated in the headspace per unit weight of soil within a unit time. The  $\text{N}_2\text{O}$  and  $\text{CO}_2$  production rates were calculated with consideration of their dissolution in the soil slurry (see Chapter 2).

### **Data Analysis**

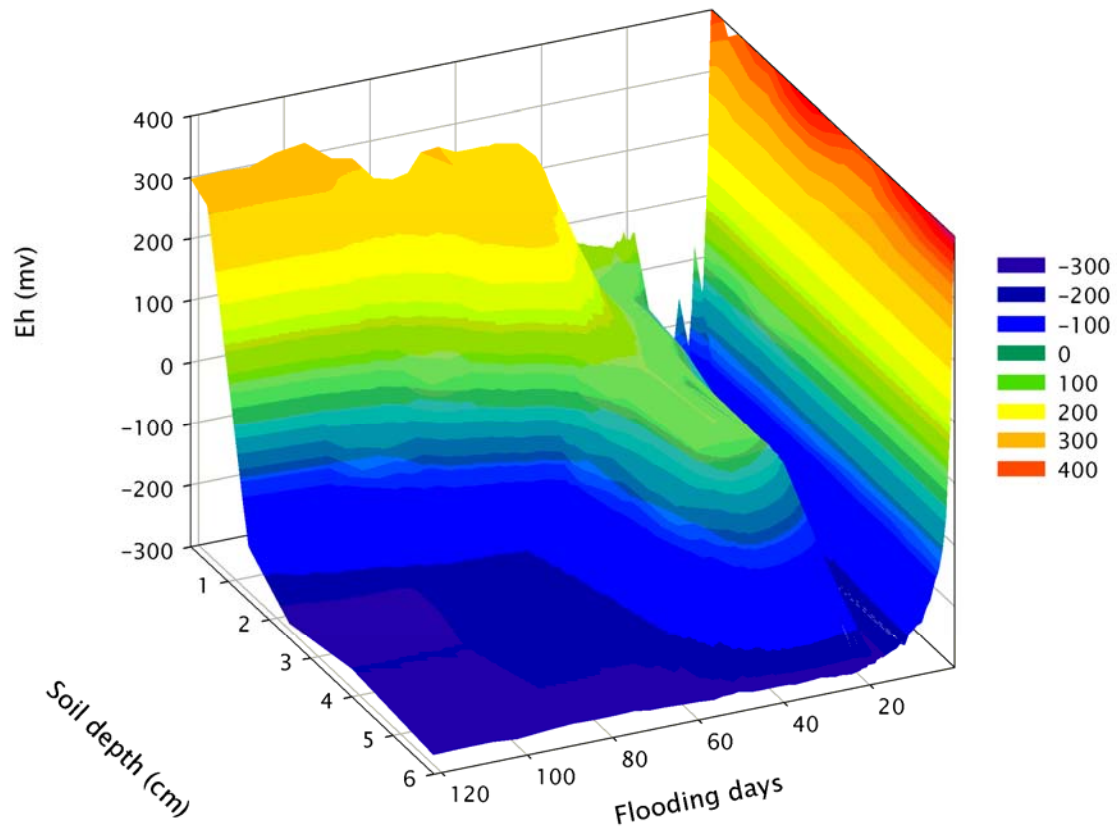
Statistical analysis was conducted with SAS (SAS Institute Inc., 1999-2001). Student t test was used for comparing production of  $\text{CH}_4$  and  $\text{CO}_2$  the soil column between treatments, depths or dates. The statistical level of significance was chosen at  $\alpha = 0.05$  for all statistical analysis.

## **Results**

### **Redox Profile before and after Nitrate Diffusion**

The soil column developed highly reducing conditions (i.e.,  $< -200$  mV) within a few days after flooding (Fig. 3.1). On day 10, even the Eh at the 0.1-cm depth reached around -200 mV. Influenced by  $\text{NO}_3^-$  diffusion on day 17, the Eh in the 0.5-4 cm layer quickly increased to and was maintained around 0 mV until day 35. Thereafter, the Eh above the 1.5-cm depth continued to rise to and remained in the weakly reducing range (i.e., 200 to

350 mV) until day 50 when the Eh at the 1.5-cm depth started to drift slowly to below -200 mV on day 120. The Eh at the 2.5- and 4-cm depths remained stable for a few days, and then decreased similarly to below -200 mV on day 60. Nitrate diffusion had little effect on the Eh at the 6.0-cm depth. Overall, the intermediately reducing zone (i.e., -100 to +150 mV) was rapidly expanded soon after  $\text{NO}_3^-$  diffusion, and clearly visible between days 20 and 50.



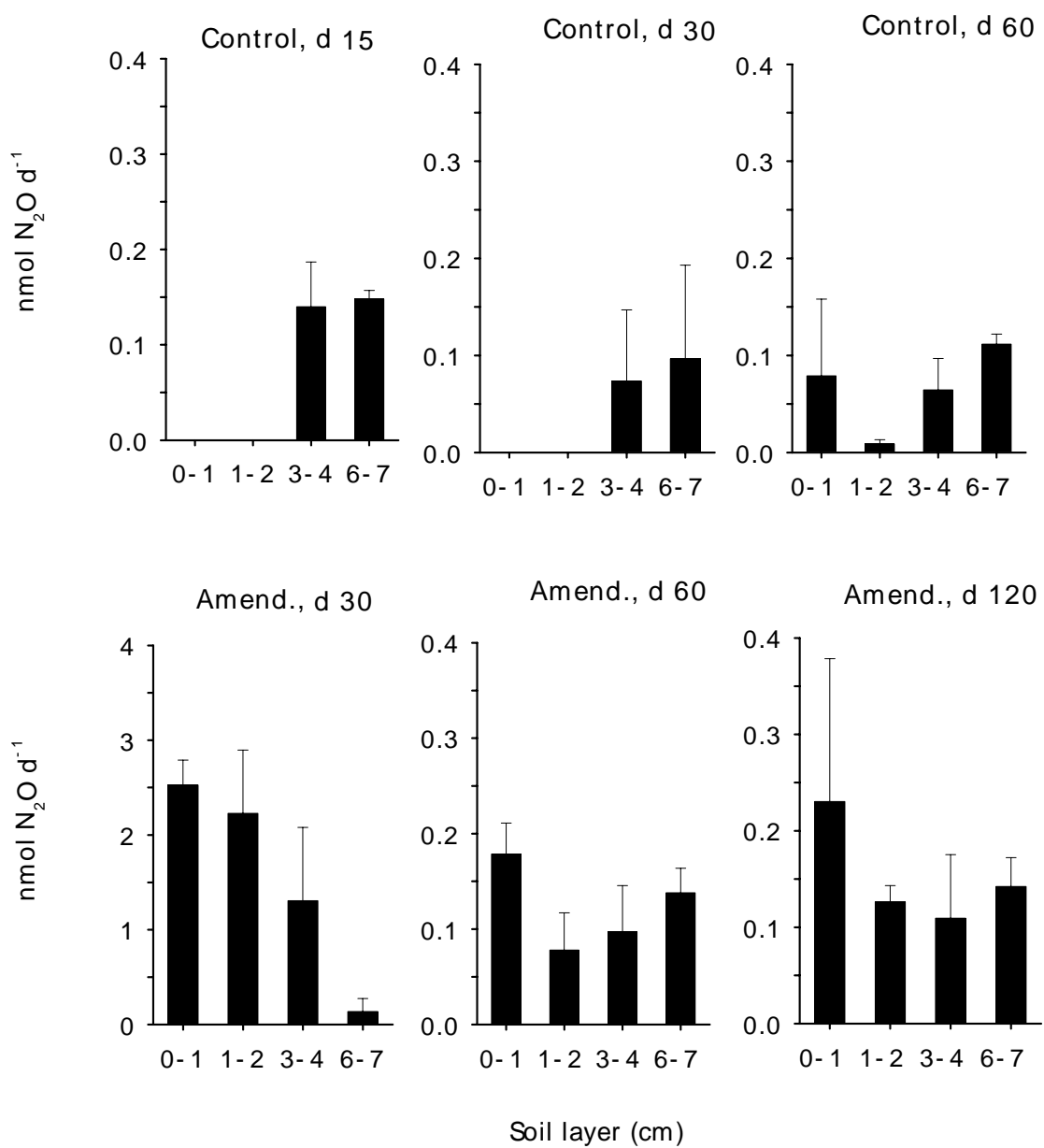
**Fig.3.1** Eh profile (n=5) in the flooded soil before and after  $\text{NO}_3^-$  addition. Nitrate was amended as potassium nitrate at 35 mM to the overlying water (2 cm deep) on day 17 after flooding.

## **Production of Nitrous Oxide, Methane and Carbon Dioxide**

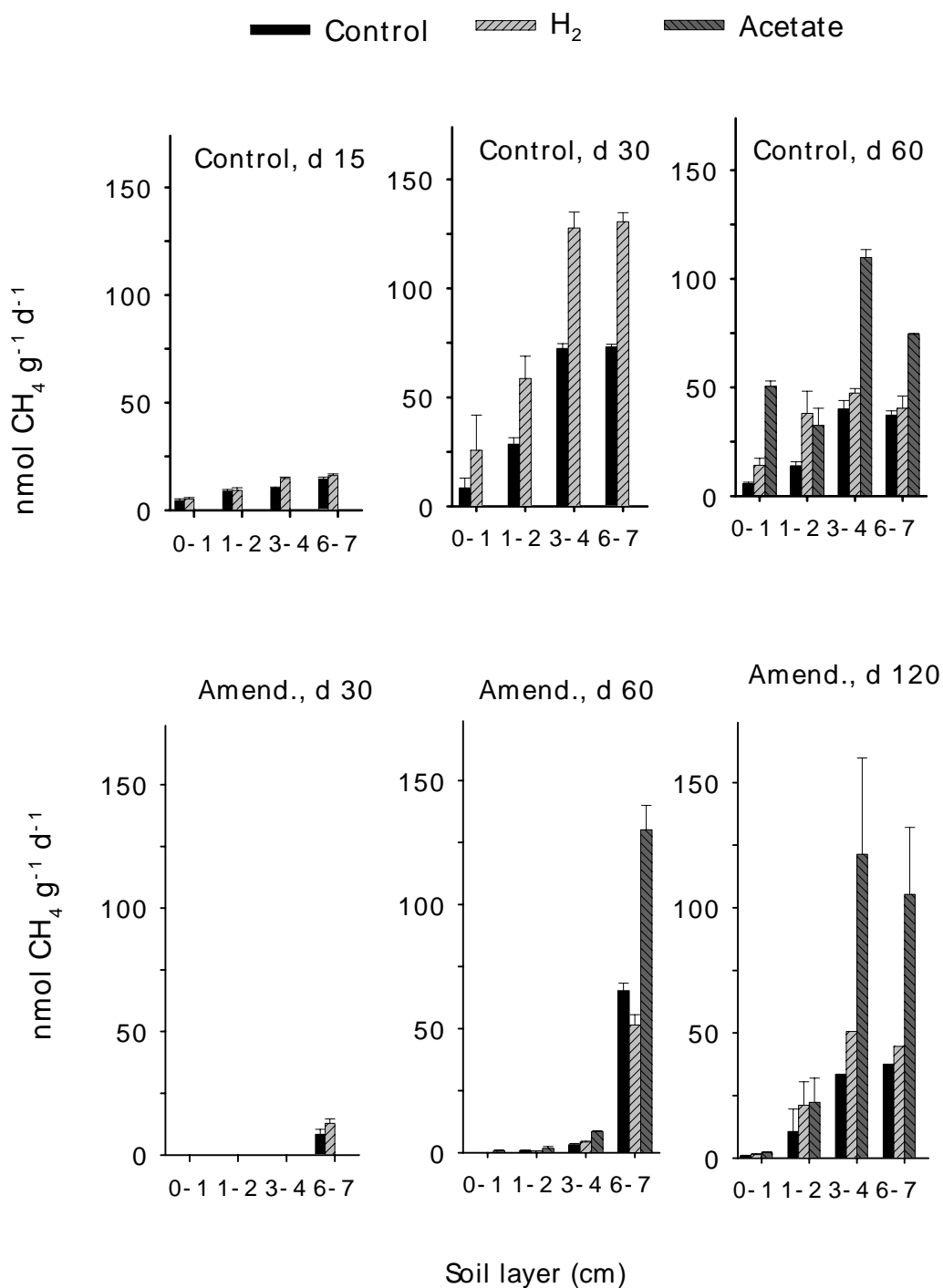
Nitrous oxide was either not detectable or produced at rates less than  $0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$  in the control soil column on each date (Fig. 3.2). Nitrous oxide production rates higher than  $1 \text{ nmol g}^{-1} \text{ d}^{-1}$  were found in the 0-1, 1-2, and 3-4 layers of the amended soil column on day 30, but were significantly lowered to around  $0.1 \text{ nmol g}^{-1} \text{ d}^{-1}$  on Day 60.

Methane production in the control soil was low on day 15 but high on days 30 and 60 except in the 0-1 cm layer (Fig. 3.3). Methane production in each layer on day 30 was significantly higher than the corresponding layer on days 15 and 60 ( $p < 0.05$ ). Under  $\text{NO}_3^-$  diffusion,  $\text{CH}_4$  production in the 6-7 cm layer was only observable on day 30 but was comparably high as found in the same layer of the control soil on day 60. Methane production was negligible or not found in the other layers of the  $\text{NO}_3^-$ -diffusing soil on days 30 and 60, but became observable in the 1-2 cm layer, and relatively high in the 3-4 cm layer on day 120.

Hydrogen-induced  $\text{CH}_4$  production in the control soil was low on day 15 but significant except in the 0-1 cm layer on day 30. On day 60,  $\text{H}_2$ -induced  $\text{CH}_4$  production was small in the 3-4 cm and 6-7 cm layers, while increased  $\text{CH}_4$  production with the acetate addition was significant relative to the control in all layers. Acetate addition also stimulated more  $\text{CH}_4$  production than by  $\text{H}_2$  addition when considerable  $\text{CH}_4$  production was found in the  $\text{NO}_3^-$ -diffusing soil, such as in the 6-7 cm layer on day 60, and in the 3-4 and 6-7 cm layers on day 120.

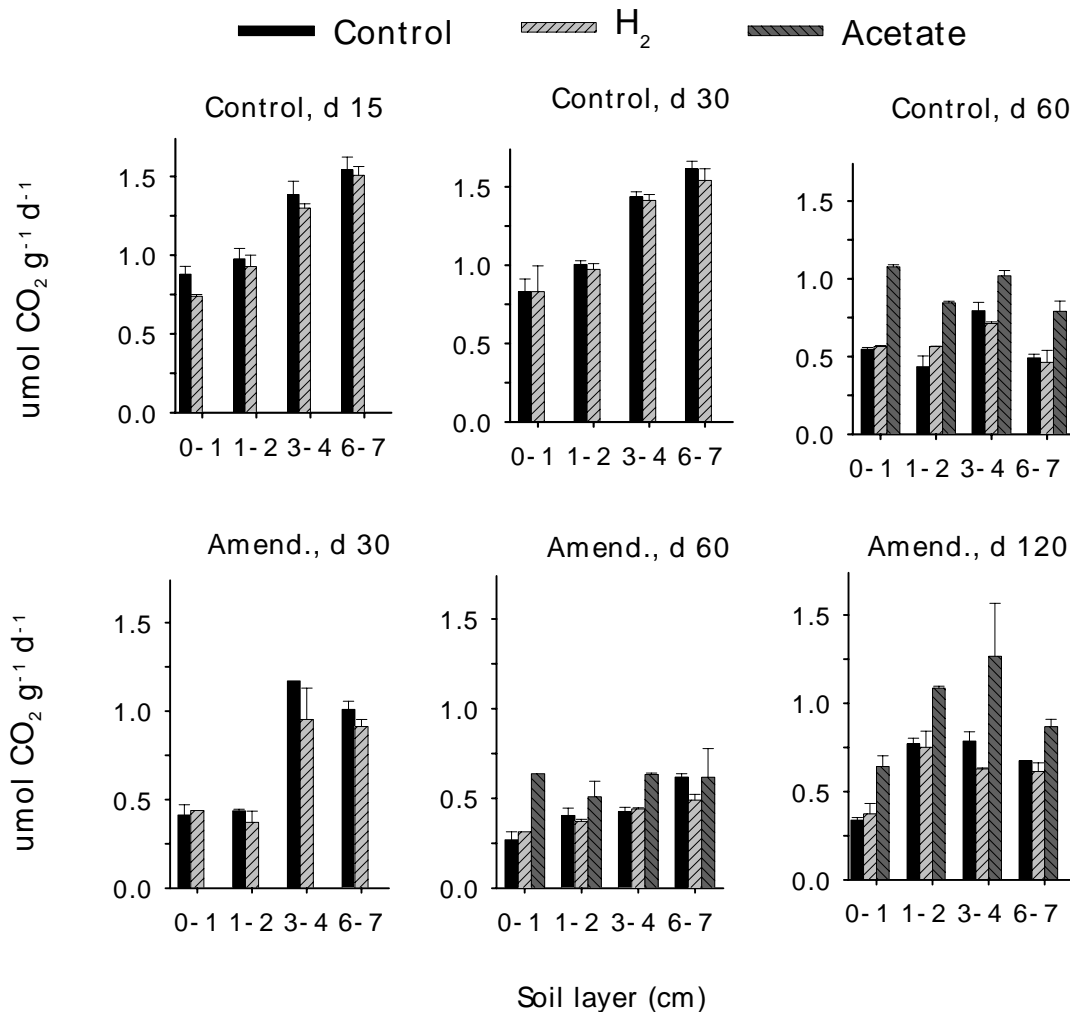


**Fig. 3.2** Nitrous oxide production (mean  $\pm$  SE,  $n=3$ ) in the control and  $\text{NO}_3^-$ -diffusing soil columns. Nitrate was amended at 35 mM to the overlying water (2 cm deep) on day 17 after flooding.



**Fig. 3.3** Methane production (mean  $\pm$  SE,  $n=3$ ) in the control and NO<sub>3</sub><sup>-</sup>-diffusing soil columns. Nitrate was amended at 35 mM to the overlying water (2 cm deep) on day 17 after flooding.

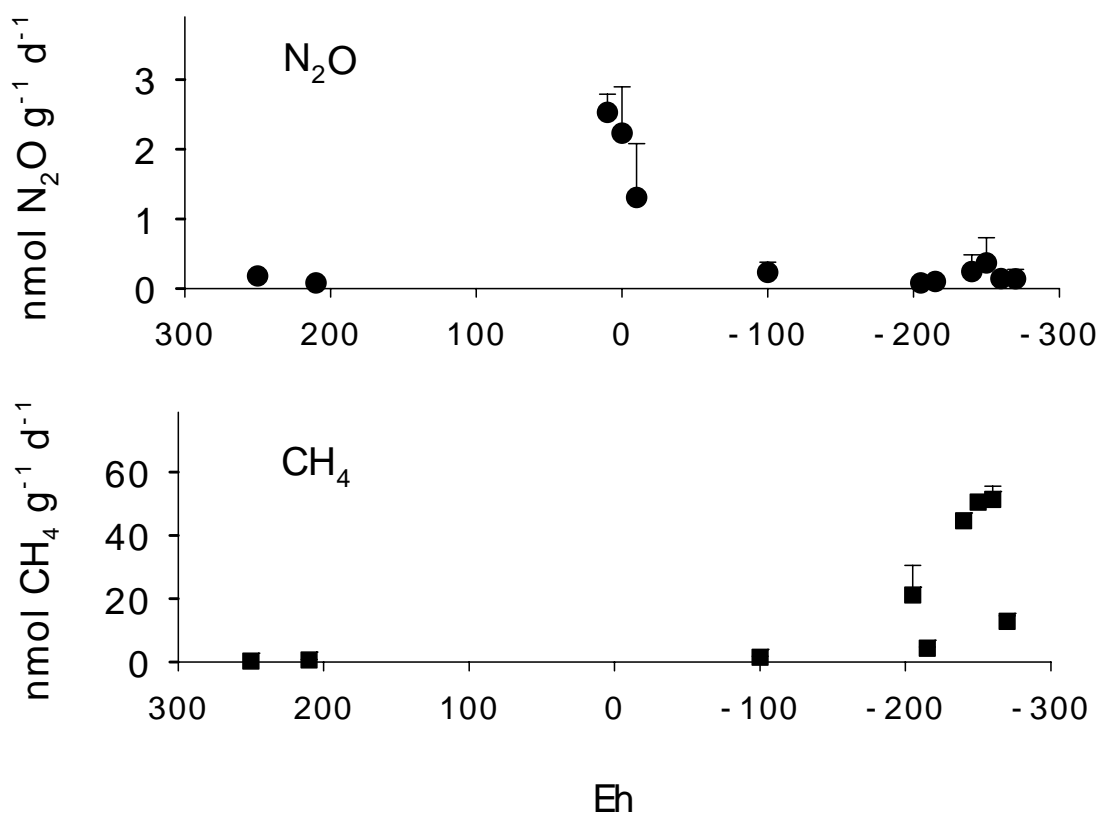
Carbon dioxide production in the two top layers was generally lower than in the two deep layers in the control soil on days 15 and 30, but became similar on day 60 (Fig. 3.4). Carbon dioxide production in the  $\text{NO}_3^-$ -diffusing soil was generally lower than in the control soil to the 7-cm depth on day 30 and to the 4-cm depth on day 60. In most cases,  $\text{CO}_2$  production was little affected by  $\text{H}_2$  addition, but was increased to some extent by acetate addition in both soil columns.



**Fig. 3.4** Carbon dioxide production (mean  $\pm$  SE,  $n=3$ ) in the control and  $\text{NO}_3^-$ -diffusing soil columns. Nitrate was amended at 35 mM to the overlying water (2 cm deep) on day 17 after flooding.

## Nitrous Oxide and Methane Production in Different Redox Ranges

Relatively high rates of  $\text{N}_2\text{O}$  production at Eh around 0 mV (Fig. 3.5) were found in the amended soil column on day 30 (Fig. 3.2), while high rates of  $\text{CH}_4$  production were only found at Eh less than -200 mV in the control and amended soil columns.



**Fig. 3.5**  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production in different Eh ranges of the control and  $\text{NO}_3^-$  diffusing soil columns.

## Discussion

### Redox Conditions

The Eh profile before  $\text{NO}_3^-$  diffusion began on day 17 indicated the control soil quickly developed highly reducing conditions. Thereafter, the Eh profile for the control

soil was not separately measured. The aerobic/anaerobic interface of the control soil may move deeper under  $O_2$  diffusion with time (Phillips et al., 1978), but would be above the 0.5-cm depth according to the Eh profile after  $NO_3^-$  diffusion and within the 0-1 cm layer since methanogenic activity was occurring in this layer on day 60. The soil layer below the 1-cm depth largely remained under highly reducing conditions through day 60.

Nitrate diffusion since day 17 temporally extended and spatially expanded the intermediate reducing zone in the soil column. Nitrate diffusion would first stimulate organic carbon consumption through heterotrophic denitrification and consequently lower carbon availability for all heterotrophic anaerobes in the soil column. This was evidenced by  $CO_2$  production in the  $NO_3^-$ -diffusing soil being generally lower than in the control soil to the 7-cm depth on day 30 and to the 4-cm depth on day 60.

Under increasing carbon limitation,  $NO_3^-$  diffused deeper to stimulate cycling of  $Fe^{3+}$ -centered AEAs in the soil column. The Eh profile showed that  $O_2$  could not reach below the 0.5-cm depth. The rapid Eh increase above the 4-cm depth after  $NO_3^-$  diffusion was primarily due to the regeneration of  $Fe^{3+}$ -centered AEAs coupling to  $NO_3^-$  reduction (Klüber and Conrad, 1998). The regenerated AEAs may be microbially reduced again but would be repeatedly recycled under the continuing influence of  $NO_3^-$  diffusion, greatly expanding and extending the intermediately reducing zone in the  $NO_3^-$ -diffusing soil. When such influence was weak or absent, the net reduction of  $Fe^{3+}$ -centered AEAs occurred, as indicated by the restart of Eh decrease at the 2.5- and 4.0-cm depths since day 40 and at the 1.5-cm depth since day 55.



## Nitrous Oxide Production

Due to the rapid depletion of  $\text{NO}_3^-$  in the soil soon after flooding,  $\text{N}_2\text{O}$  production was either undetectable or negligible in the control soil. Nitrate diffusion dramatically induced  $\text{N}_2\text{O}$  production at Eh around 0 mV above the 4-cm depth on day 30. High  $\text{N}_2\text{O}$  production around 0 mV was also observed under Eh-controlled slurries (Kralova et al., 1992). The Eh increase above the 4-cm depth during this period indicated  $\text{Fe}^{3+}$ -centered AEAs were under intensive regeneration. Such high  $\text{N}_2\text{O}$  production was not only ascribed to intensified denitrification, but also due to the effect on  $\text{N}_2\text{O}$  production of  $\text{Fe}^{2+}$  reoxidation (Chapter 2). While such intensive interaction of  $\text{Fe}^{2+}$  with  $\text{NO}_3^-$  reduction could not easily occur in soil slurries where redox processes were largely sequentially initiated (Chapter 2), it can be found in  $\text{NO}_3^-$ -polluted aquatic environments and even in the rhizosphere. Such interaction not only affect the fate of  $\text{NO}_3^-$  including  $\text{N}_2\text{O}$  production, but may also affect the stability of heavy metals associated with the consequently changed iron-centered intermediate redox processes (Senn et al., 2002).

## Methanogenesis

While the soil layer below the 1-cm depth largely remained under highly reducing conditions through day 60 in the control soil, cycling of  $\text{Fe}^{3+}$ -centered AEAs, occurring around the anaerobic/anaerobic interface and largely mediated by  $\text{O}_2$  diffusion (Frenzel et al., 1999; Rysgaard et al., 2001), was effective in lowering electron donor availability for anaerobic activity, especially methanogenic activity above the 2-cm depth.

The importance of electron donor availability in controlling anaerobic microbial activity was clearly revealed by substrate-induced  $\text{CH}_4$  and  $\text{CO}_2$  production at different times. In the early period (on day 15),  $\text{H}_2$  addition did not significantly increase  $\text{CH}_4$  production, mainly because methanogenic activity was weak while more competitive redox processes such as  $\text{Fe}^{3+}$  reduction may still continue. During the mid- and late-periods when methanogenic activity was sustained, the addition of substrate, especially acetate, significantly stimulated  $\text{CH}_4$  formation, even below the 3-cm depth where strong methanogenesis was found. Thus, substrate availability would largely control the vertical and temporal variations of methanogenesis in these periods. Meanwhile, the addition of hydrogen or acetate may also stimulate other anaerobic microbial activity besides methanogenesis. Only  $\text{H}_2$  induced microbial activity was not directly reflected by changes in  $\text{CO}_2$  production. Acetate-stimulated  $\text{CO}_2$  production, e.g. in the 0-1 and 1-2 cm layer on day 60, was not only derived from  $\text{CO}_2$  produced in methanogenesis but also from anaerobic oxidation of acetate by other anaerobes such as  $\text{Fe}^{3+}$  reducers (Chidthaisong and Conrad, 2000).

Methanogenic activity in the  $\text{NO}_3^-$ -diffusing soil was greatly lowered, not only due to increased carbon limitation and  $\text{NO}_3^-$  inhibition, but also due to the suppressing effect of regeneration of  $\text{Fe}^{3+}$ -centered AEAs. While the regenerated AEAs under  $\text{NO}_3^-$  diffusion, such as  $\text{Fe}^{3+}$  as an inhibitor on methanogens, were not as strong as  $\text{NO}_3^-$  and its intermediate products  $\text{NO}$  and  $\text{N}_2\text{O}$  (Chidthaisong and Conrad, 2000), they were strong enough to suppress methanogenic activity under carbon-limiting conditions (Achthich et

al., 1995). The 0-1 cm layer remained under weakly reducing conditions due to the strong influence of  $\text{NO}_3^-$  diffusion and prevented methanogenesis until day 120. The 1-2 and 3-4 cm layers underwent intensified cycling of  $\text{Fe}^{3+}$ -centered AEAs under  $\text{NO}_3^-$  diffusion, resulting in substantial reduction of methanogenesis even after these layers had re-developed highly reducing conditions. These intensified intermediate redox processes even effectively lowered the availability of electron donors in the highly reducing 6-7 cm layer, resulting in significant reduction of methanogenesis for at least 13 days.

The effect of cycling of  $\text{Fe}^{3+}$ -centered AEAs on methanogenesis observed in both control and  $\text{NO}_3^-$ -diffusing soil columns shows the need of incorporating the role of cycling of  $\text{Fe}^{3+}$ -centered AEAs in prediction of methanogenesis in wetland soils. The consideration of cycling of  $\text{Fe}^{3+}$ -centered AEAs is still preliminary in recent model studies on prediction of  $\text{CH}_4$  emission in rice paddies (Matthews et al., 2000).

## **Conclusions**

Cycling of  $\text{Fe}^{3+}$ -centered AEAs in the control flooded soil, primarily regulated by the diffusion of  $\text{O}_2$ , was restricted around the aerobic/anaerobic interface but effectively suppressed methanogenesis above the 2-cm depth. Their cycling was vertically expanded and temporally extended under  $\text{NO}_3^-$  diffusion. Subsequently, primarily through lowering carbon source availability to methanogens, the intensified cycling of  $\text{Fe}^{3+}$ -centered AEAs resulted in significant suppression of methanogenesis to deeper depths for longer periods. Nitrous oxide production in the control soil was negligible due to  $\text{NO}_3^-$  depletion. High  $\text{N}_2\text{O}$  production occurred even at Eh around 0 mV under  $\text{NO}_3^-$  diffusion, partly due to

intensive interaction of  $\text{Fe}^{2+}$  reoxidation with  $\text{NO}_3^-$  reduction. The effect of cycling of  $\text{Fe}^{3+}$ -centered AEAs on the temporal and vertical variations of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production in the flooded soil shows the importance of cycling of  $\text{Fe}^{3+}$ -centered AEAs in evaluation and predication of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  formation in wetland and aquatic environments.

## **CHAPTER 4**

### **FATE OF NITRATE IN A FLOODED RICE SOIL AFFECTED BY POOL OF ALTERNATIVE ELECTRON ACCEPTORS**

#### **Introduction**

Increasing N fertilizer applications to agricultural lands, including rice fields, causes serious  $\text{NO}_3^-$  pollution in surface and subsurface water bodies (Aulakh and Singh, 1997; Ghosh, 1998). Natural and constructed wetlands are a sink for  $\text{NO}_3^-$  from waters (Hill, 1996; Reilly et al., 2000; Rutherford and Nguyen, 2004). In wetland environments, the biologically active zone is the functioning zone largely responsible for  $\text{NO}_3^-$  transformation. Pools of organic carbon sources and oxidative minerals and water movement in this functioning zone are major factors determining the ability of wetlands to remove  $\text{NO}_3^-$  from waters. The availability of organic carbon sources is important in influencing the rate of  $\text{NO}_3^-$  transformation (Weisner et al., 1994; Ingersoll and Baker, 1998; Hill and Cardaci, 2004) while water movement controls the contact time for  $\text{NO}_3^-$  transformation in the functioning zone. Highly reducing conditions may be formed in part of the functioning zone with organic carbon inputs from aquatic plants. A deep highly reducing zone is able to efficiently remove  $\text{NO}_3^-$  (Kralova et al., 1992) within a range of water residence times.

Nevertheless, the importance of the pool of oxidative minerals in  $\text{NO}_3^-$  removal by wetlands should also be appreciated, especially when wetlands are seasonally and intermittently flooded or the overlying water is under intensive fluctuation (e.g. riparian

wetlands and rice fields). The capacity of the functioning zone for  $\text{NO}_3^-$  removal under such conditions may be lowered due to intensive aerobic oxidation, lack of organic carbon inputs and intensive generation and regeneration of  $\text{Fe}^{3+}$ -centered AEAs. Simultaneous biological removal of  $\text{NO}_3^-$  in the process of  $\text{NO}_3^-$  transport may restrict  $\text{NO}_3^-$  availability within certain parts of the functioning zone for certain periods while microbial reduction of other AEAs such as  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  may occur in other parts of the functioning zone. Substantial microbial reduction of these AEAs may lower the availability of carbon sources to heterotrophic denitrification in the functioning zone to promote  $\text{N}_2\text{O}$  formation and  $\text{NO}_3^-$  transport. As a result, additional  $\text{N}_2\text{O}$  emission from and  $\text{NO}_3^-$  transport through the functioning zone may occur (Staver and Brinsfield, 1996; Zhu et al., 2000; Dowling et al., 2004).

Oxidized manganese and iron minerals are a major component of the pool of AEAs in most freshwater wetlands (Yao et al., 1996). While the impact of microbial  $\text{Fe}^{3+}$  reduction on denitrification, including  $\text{N}_2\text{O}$  production, was observed in the soil slurry experiment (Chapter 2), the significance of this impact under field conditions is yet to be carefully evaluated. As a closer step to more realistic conditions, in this study we used flooded soil columns to study the effects of amendments of oxidative iron and manganese oxides on fate of  $\text{NO}_3^-$  in a rice soil with and without percolation.

## Materials and Methods`

### Soil Column Incubation

A Crowley silt loam soil was used in the experiment (Chapter 2). Polyvinyl Chloride (PVC) columns (5.5 x 25 cm), with one end capped, were used for the flooded soil column incubations. One set (n=3) of the PVC columns were used for the non-percolation experiment and another set (n=3), being installed with a 2-cm layer of glass wool and quartz sand at the bottom and an outlet close to the bottom, were used for the percolation experiment. Four hundred g of air-dried soil was mixed with or without MnO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> powder (2.7 and 5.0 mg g<sup>-1</sup>, respectively), and packed into each PVC column to form a 12-cm soil column. The soil columns were flooded with deionized water to form a 4.5-cm layer of overlying water and incubated at 22°C in the dark. Potassium nitrate (0.1 g NO<sub>3</sub><sup>-</sup>-N) was amended to the overlying water (equivalent 70 mM NO<sub>3</sub><sup>-</sup>) of the column 1 day after flooding, and then 10 ml percolate was collected daily by a syringe connected to the bottom of the soil column (equivalent to a moderate percolation rate of 4.2 mm d<sup>-1</sup> in irrigated rice fields) and frozen until NO<sub>3</sub><sup>-</sup> content measurement. Deionized water was added daily after percolate collection to maintain the depth of the overlying water at 4.5 cm for the percolated and unpercolated soil columns. The collection of percolates stopped at week 7 when the NO<sub>3</sub><sup>-</sup> content in the percolate was found to be below detection at week 6. The incubation of the unpercolated soil columns continued until week 16 for the measurement of NO<sub>3</sub><sup>-</sup> content remaining in the overlying water.

## **Redox Profile**

The Eh profile in the 0-10 cm layer of the soil column was measured at 0.5-cm (near surface) or 1.0 cm increments at weeks 2, 4, 6 and 8 after  $\text{NO}_3^-$  addition with a platinum electrode modified from that described by Patrick et al. (1996). Each time before measurement, the platinum electrode was tested using quinhydrone-saturated standard pH solutions (Patrick et al., 1996). A silver chloride reference electrode was used during Eh measurement. The Eh readings were recorded 15 min after the electrode reached a specific depth, and reported after correction against the standard  $\text{H}_2$  electrode by adding 200 mV.

## **Nitrous Oxide Flux**

Nitrous oxide fluxes in the soil columns ( $n=2$ ) were measured on days 1, 3, 7, 14, 28, and 42 after  $\text{NO}_3^-$  addition. After flushing with ambient air, the column was capped with a rubber stopper which was fitted with a septum. Gas samples of 10 ml were taken from the headspace at 0 and 30 min after closing and analyzed with a Tremetrics 9001 gas chromatograph using an electron capture detector (ECD) for determining  $\text{N}_2\text{O}$  concentrations.

## **Nitrate Content in Percolate and in Overlying Water**

The frozen percolate samples were thawed at  $4^\circ\text{C}$ . The samples collected from the column ( $n=2$ ) in each week were combined as one. Five ml of the combined percolate was filtered and stabilized with 5 ml buffer solution (APHA, 1995) for  $\text{NO}_3^-$  content determination with an ion meter and a  $\text{NO}_3^-$ -selective electrode (detection limit  $7 \times 10^{-6}$



M). Five ml of water was directly sampled from the overlying water at week 6 for the percolated soil columns and at the end of the incubation (week 16) for the unpercolated soil columns and stabilized with 5 ml buffer solution for  $\text{NO}_3^-$  content determination.

### **Data Analysis**

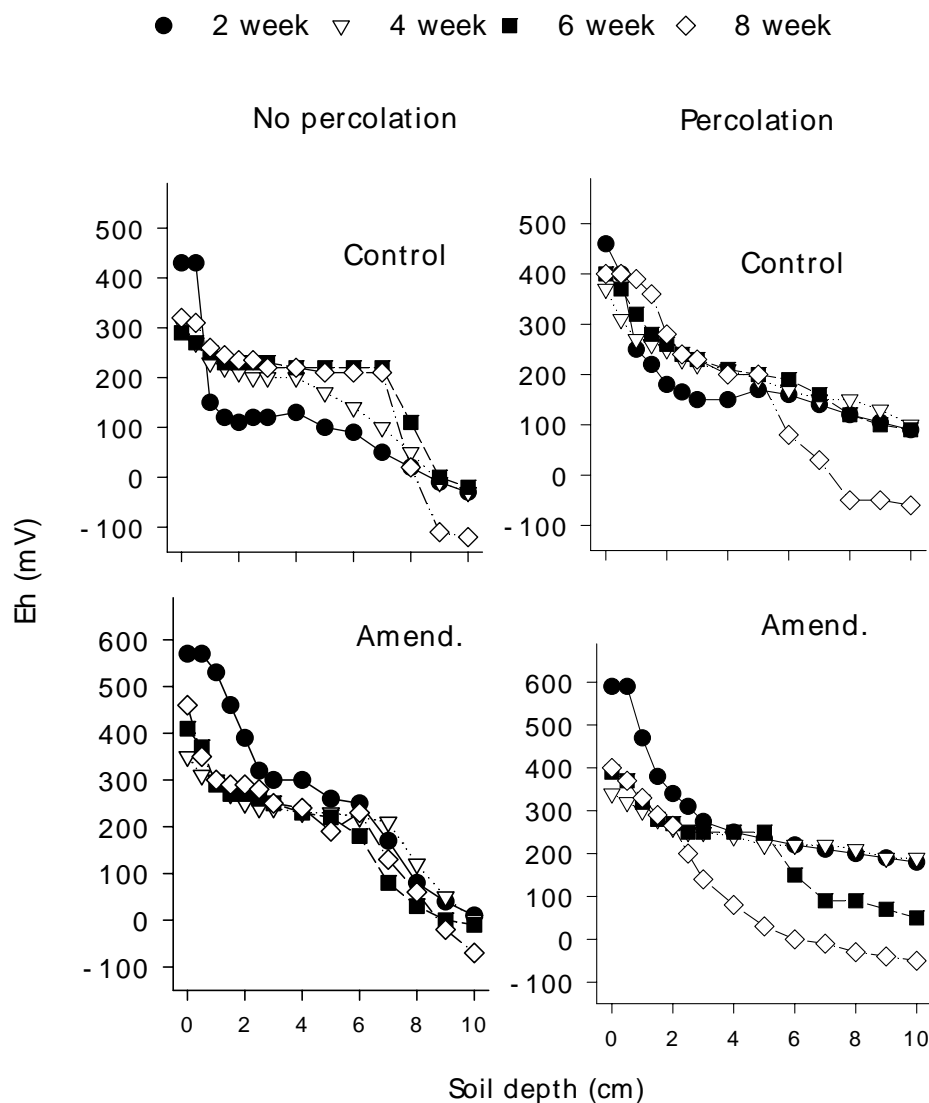
Statistical analysis was conducted with SAS (SAS Institute Inc., 1999-2001).

Variations in  $\text{N}_2\text{O}$  emission and  $\text{NO}_3^-$  percolation between treatments were analyzed with one-way analysis of variance. The statistical level of significance was chosen at  $\alpha = 0.05$  for all statistical analysis.

## **Results**

### **Redox Profile**

Different redox profiles were observed in the control and amended soil columns (Figure 4.1). The control soil developed intermediate reducing conditions at the 1-7 cm depths at week 2 and weakly reducing conditions from week 4 to 8. The amended treatment maintained weakly to intermediately reducing conditions at the 1-7 cm depths from week 2 to 8. The redox discontinuity (a rapid change in Eh slope before and after a depth) in the control soil, occurring around the 7-cm depth at week 8, was sharper than in the amended soil. Percolation greatly flattened the redox profiles in both treatments in the first 6 weeks. At week 8 the redox discontinuity zone was moved to the 5-cm depth in the control treatment and the 4-cm depth in the amended treatment.

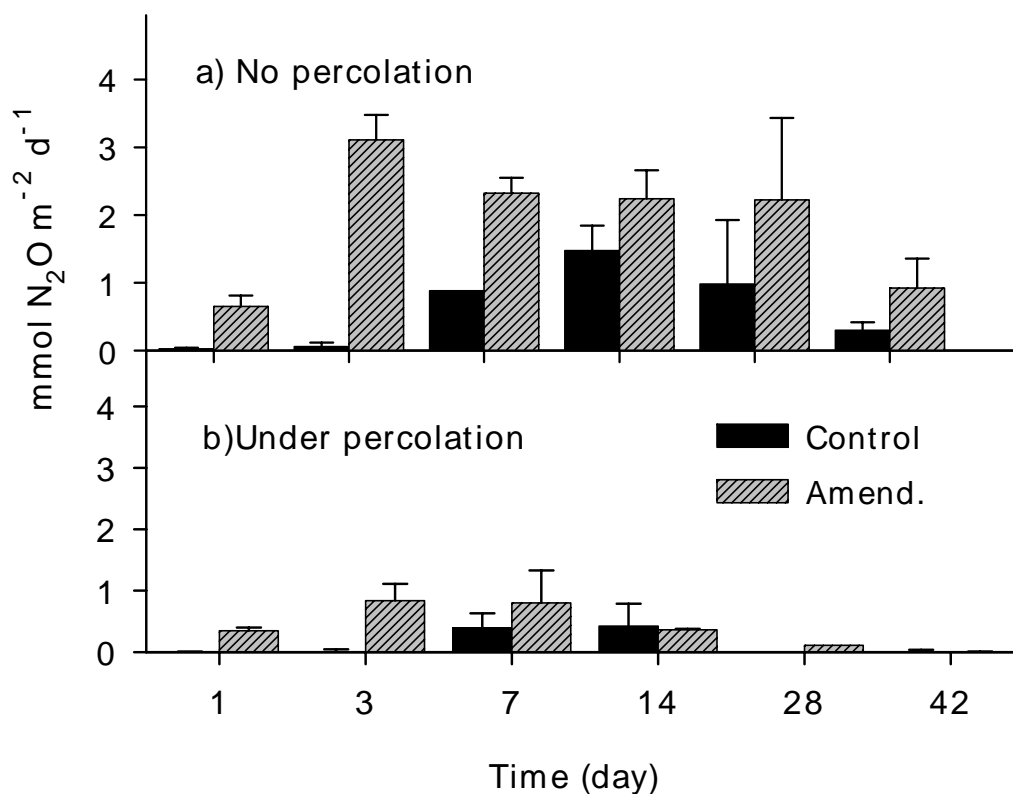


**Fig. 4.1** Redox profiles in the flooded soil columns affected by amendments of  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  and percolation. The soil columns were amended with potassium nitrate ( $0.1 \text{ g NO}_3^- \text{-N}$ ) to the 4.5-cm overlying water (equivalent to  $70 \text{ mM NO}_3^-$ ) 1 day after flooding, with or without amendments of  $\text{MnO}_2$  and  $\text{Fe}_2\text{O}_3$  powder before flooding ( $2.7$  and  $5.0 \text{ mg g}^{-1}$ , respectively), and percolated at  $0$  or  $4.2 \text{ mm d}^{-1}$ .

### Nitrous Oxide Emission

Without percolation,  $\text{N}_2\text{O}$  flux was higher in the amended treatment than in the control treatment on each date until day 42 when  $\text{N}_2\text{O}$  emission measurement stopped (Fig. 4.2). Percolation significantly reduced  $\text{N}_2\text{O}$  emissions in both treatments. The  $\text{N}_2\text{O}$

emission in the 42-day measurement was significantly increased by amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  in both unpercolated and percolated soil columns ( $p < 0.05$ ) (Table 4.1).

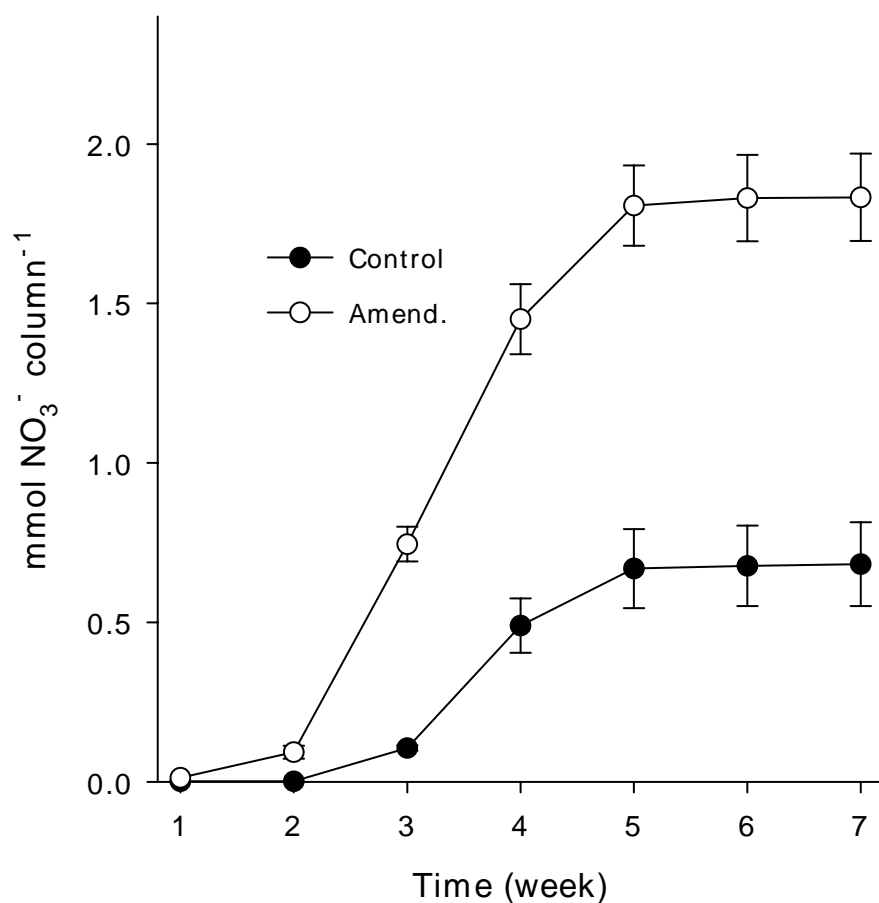


**Fig. 4.2** Fluxes of  $\text{N}_2\text{O}$  in the flooded soil columns affected by amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  and percolation (mean  $\pm$  SE,  $n=2$ ). For explanation, see Fig. 4.1.

### Nitrate Transformation and Transport

Under no percolation, the levels of  $\text{NO}_3^-$  remaining in the overlying water at week 16 were 12.0 mM in the control treatment and 22.8 mM in the amended treatment, accounting for 17.2 and 32.5% of  $\text{NO}_3^-$  applied, respectively. Under percolation, the levels of  $\text{NO}_3^-$  remaining in the overlying water at week 6 were 0.7 and 0.9 mM in the control and amended treatments, respectively. Nitrate percolation in the control treatment

compared to the amended treatment was significantly lower ( $p < 0.01$ ) and started 1 week later (Fig. 4.3). Nitrate removal by percolation mainly occurred between weeks 3 to 5 in both treatments. The 6-week cumulative amounts of  $\text{NO}_3^-$  percolated were 10.9 and 24.3% of  $\text{NO}_3^-$  applied in the control and amended treatments, respectively. Without considering the amounts of  $\text{NO}_3^-$  remaining within the flooded soils, the averaged rates of  $\text{NO}_3^-$  transformation were higher in both treatments under percolation (Table 4.1).



**Fig. 4.3** Cumulative  $\text{NO}_3^-$  percolation from the flooded soil column affected by amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$ . For explanation, see Fig. 4.1.

**Table 4.1** Fate of  $\text{NO}_3^-$  in the flooded soil as affected by amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$ . The soil columns were amended with potassium nitrate ( $0.1 \text{ g NO}_3^- \text{-N}$ ) to the 4.5-cm overlying water (equivalent to  $70 \text{ mM NO}_3^-$ ) 1 day after flooding, with or without amendments of  $\text{MnO}_2$  and  $\text{Fe}_2\text{O}_3$  powder before flooding ( $2.7$  and  $5.0 \text{ mg g}^{-1}$ , respectively), and percolated at  $0$  or  $4.2 \text{ mm d}^{-1}$ .

Percolation ( $\text{mm d}^{-1}$ )	Treatment	Duration (d)	$\text{NO}_3^-$ removal ( $\text{mmol m}^{-2} \text{ d}^{-1}$ )	$\text{NO}_3^-$ percolated ( $\text{mmol m}^{-2} \text{ d}^{-1}$ )	$\text{N}_2\text{O}$ emission ( $\text{mmol m}^{-2} \text{ d}^{-1}$ )
0	Control	112	23.3	-	0.87
0	Amend.	112	19.0	-	2.01
4.2	Control	42	66.1	8.1	0.16
4.2	Amend.	42	55.8	18.2	0.30

## Discussion

Oxygen in the soil columns was depleted soon after flooding. Oxygen diffusion might only have a direct effect on microbial activity in the surface layer of the soil columns even under percolation according to the redox profile. The size and composition of the pool of AEAs became important in determination of the succession of anaerobic redox processes in the flooded soil columns. The amendments of  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  increased the pool of AEAs and consequently changed redox conditions and  $\text{NO}_3^-$  fate in the soil columns.

## Redox Profile

The development of redox conditions at a specific depth in the soil columns was microbially mediated and controlled by the availability of AEAs relative to electron donors. The  $\text{NO}_3^-$  diffusion plume stimulated heterotrophic denitrification increasing carbon source limitation (see Chapter 4). The downward diffusion of  $\text{NO}_3^-$  also promoted

the regeneration of AEAs, increasing Eh from week 2 to 4 at the 2-7 cm depths in the control soil column. The amendments of iron and manganese oxides, increasing the pool of AEAs, further increased carbon limitation for denitrification and greatly delayed Eh changes at the 1-8 cm depths from week 2 to 8. Under percolation,  $\text{NO}_3^-$  was transported much deeper, resulting in greatly flattened redox profiles in both treatments in the first 6 weeks.

### **Fate of Nitrate**

Soil physical properties such as texture are important in controlling  $\text{NO}_3^-$  transport in flooded soils. Nevertheless, in the same soil and under a specific percolation rate, the availability of electron donors and  $\text{Fe}^{3+}$ -centered intermediate AEAs largely controlled the ability of the soil to transform (mainly denitrify)  $\text{NO}_3^-$  and  $\text{NO}_3^-$  transport by diffusion and percolation. Dissimilatory reduction of  $\text{NO}_3^-$  to ammonium, occurring when electron donor availability is high (King and Nedwell, 1985), was not expected to be an important pathway for  $\text{NO}_3^-$  transformation in this experiment.

Indigenous soil  $\text{NO}_3^-$  was depleted soon after flooding in these incubations. Nitrate transport from the overlying water to the soil should not affect the reduction of  $\text{Mn}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$ , and/or  $\text{CH}_4$  formation in the deep soil layer immediately. So, before  $\text{NO}_3^-$  reached a deeper depth, some readily available carbon sources had been consumed by  $\text{Fe}^{3+}$  reducers, etc. The amount of available carbon at a specific soil depth consumed by anaerobes other than heterotrophic denitrifiers was dependent on the time needed for  $\text{NO}_3^-$  to reach that depth and the availability of other AEAs such as  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  at that

depth. With greater time and more AEAs, more available carbon would be consumed to limit heterotrophic denitrification and promote  $\text{N}_2\text{O}$  emission and  $\text{NO}_3^-$  transport.

Under no percolation,  $\text{NO}_3^-$  still remained in the overlying water at week 16. Passive  $\text{NO}_3^-$  diffusion appeared to be slow and could not reach below the 8-cm depth according to the redox profile while the upper soil layer (e.g. 0-2 cm depths) underwent continuous  $\text{NO}_3^-$  transformation with  $\text{N}_2\text{O}$  being emitted as the end product at high rates under increasing carbon limitation (Benckiser et al., 1996). The amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$ , increasing the pool of AEAs, further increased the limitation of carbon source to heterotrophic denitrifiers. While the amendments of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  were expected to intensify redox cycling of  $\text{Mn}^{4+}$  and  $\text{Fe}^{3+}$  to promote chemical and/or chemo-autotrophic  $\text{NO}_3^-$  reduction, this effect appeared to be less efficient than heterotrophic denitrification, decreasing  $\text{NO}_3^-$  transformation overall to promote more  $\text{N}_2\text{O}$  emission.

The effect of amendments of  $\text{Fe}^{3+}$  and  $\text{Mn}^{4+}$  on  $\text{NO}_3^-$  transformation was also evidenced in the percolated soil column. Although the redox profiles were quite similar in the first 6 weeks and microbial reduction of  $\text{Fe}^{3+}$ -centered AEAs would be suppressed in the whole soil columns of both treatments from week 3 to 5 when  $\text{NO}_3^-$  percolation mainly occurred, the amendments of iron and manganese oxides were still effective in lowering  $\text{NO}_3^-$  transformation while increasing  $\text{NO}_3^-$  percolation. The significant reductions in  $\text{N}_2\text{O}$  emission under percolation in both treatments were ascribed to high solubility of  $\text{N}_2\text{O}$  in water (Handbook of Chemistry and Physics, 1991).

It should be noted that while percolation increased the risk of  $\text{NO}_3^-$  transport to deeper groundwater, it also promoted  $\text{NO}_3^-$  removal in both control and amended soil columns through promoting deeper transport of  $\text{NO}_3^-$  which increased the effectiveness of the flooded soil column for  $\text{NO}_3^-$  removal. While it is possible that strong percolation decreases  $\text{NO}_3^-$  removal through dramatically shortening the residence time, it is more likely that percolation at slow to moderate rates increases the efficiency of the functioning zone to remove  $\text{NO}_3^-$  under field conditions. Besides the pool of organic carbon sources (Weisner et al., 1994; Ingersoll and Baker, 1998; Hill and Cardaci, 2004), the availability of AEAs other than  $\text{NO}_3^-$  and water movement need to be considered with the processes of groundwater discharge and recharge for  $\text{NO}_3^-$  removal by wetlands, specifically under seasonal and intermittent flooding conditions. Such considerations have been limited in evaluating  $\text{NO}_3^-$  removal by treatment wetlands and  $\text{NO}_3^-$  leaching/percolation in rice paddies (Zhu et al., 2000). More studies are needed on the interrelations between pools of carbon sources and AEAs and water movement in  $\text{NO}_3^-$  removal and  $\text{N}_2\text{O}$  emission in treatment wetlands.

### **Conclusions**

The flooded soil column was a strong sink for  $\text{NO}_3^-$  but also a strong source of  $\text{N}_2\text{O}$  under simulated conditions of elevated nitrate in the overlying water. Simultaneous biological  $\text{NO}_3^-$  removal during the slow process of  $\text{NO}_3^-$  passive diffusion restricted  $\text{NO}_3^-$  within the upper part of the soil column. Percolation at a moderate rate resulted in the occurrence of  $\text{NO}_3^-$  percolation but also promoted  $\text{NO}_3^-$  removal and lessened  $\text{N}_2\text{O}$



emission, through promoting deep transport of  $\text{NO}_3^-$  to increase the effectiveness of the soil column for  $\text{NO}_3^-$  removal.

An increase in pool of AEAs through amendments of iron and manganese oxides increased the limitation of carbon sources, especially in the deep layer, to heterotrophic denitrification and hence lowered  $\text{NO}_3^-$  transformation, but increasing AEAs promoted  $\text{N}_2\text{O}$  formation and  $\text{NO}_3^-$  transport. Besides the availability of organic carbon sources, the pool of AEAs and percolation (more generally water movement) need to be considered to predict  $\text{NO}_3^-$  removal and  $\text{N}_2\text{O}$  emission in wetlands, especially under seasonal and intermittent flooding conditions.

## **CHAPTER 5**

### **MICROBIAL ACTIVITY IN DIFFERENT REDOX ZONES OF A FLOODED SOIL**

#### **Introduction**

Most redox processes in anaerobic environments are microbially mediated.

Assessment of microbial activity under different redox conditions is important in understanding of biogeochemical cycling of natural and pollutant substances in anaerobic environments. Redox gradients formed in anaerobic environments are normally too steep, from aerobic to weakly reducing through intermediately reducing to highly reducing conditions within a distance from a few millimeters to a few centimeters, to carry out in situ measurements of microbial activity relating to specific redox processes under specific redox conditions. It is even more difficult to estimate microbial activity in iron cycling-centered intermediately reducing zones due to their normally transitional occurrence in many anaerobic environments. In a few environments, e.g., subsurface soils, intermediately reducing zones are found as long flat redox profiles, due to an excess of iron-centered intermediate AEAs relative to electron donors (Ludvigsen et al., 1998). Flat redox profiles may be also formed in surface soils under certain conditions. Low levels of soil organic carbon, abundant presence of one or more types of alternative electron acceptors (e.g.  $\text{NO}_3^-$  and  $\text{Fe}^{3+}$ ) and low temperatures may all create flat redox profiles in wetland and aquatic environments.

The redox potential-controlled soil slurry system (Patrick et al., 1973) has been

intensively used to study redox processes under different redox conditions, including the intermediately reducing condition. The redox potential is controlled by adjustment of the availability of electron donors (e.g. glucose) and electron acceptors, normally  $O_2$ , which is toxic to anaerobes, especially obligate anaerobes (Roy et al., 1997). Redox processes have also been studied along redox gradients in anoxic soils (Ludvigsen et al., 1998), although redox conditions are rarely controlled. It is possible to study microbial activity in different redox zones of a flooded soil after the redox profile is flattened using some approach, e.g.,  $NO_3^-$  diffusion (Chapter 3).

Multiple functional groups of microorganisms, e.g., denitrifiers,  $Fe^{3+}$  reducers and methanogens are involved in microbial activity in anaerobic soils. The relative importance of these functional groups varies temporally and spatially under different redox conditions. For example, denitrification activity may dominate in a weakly reducing condition (Dassonville et al., 2004) while fermenting bacteria and methanogens are important in the methanogenic condition (Conrad, 1999). This difference needs to be considered when a specific method for microbial activity estimation is adopted.

Compared to measurements of ATP, microbial biomass, etc (Inubushi et al., 1991; Karl, 1993), measurement of glucose- or acetate-induced respiration is more widely used for microbial activity estimation in anaerobic soils. Nevertheless, their applications in different redox zones dominated by specific redox processes are rarely compared. In this study, respiration induced by acetate and glucose was comparatively evaluated for microbial activity estimation in different redox zones of a rice soil formed with and

without  $\text{NO}_3^-$  diffusion. Oxygen-induced respiration was also measured to reflect the availability of carbon sources for microbial activity in the different redox zones.

## **Materials and Methods**

### **Soil Column Incubation**

A silt loam soil was used in the experiment (see Chapter 2). PVC columns (5.5 x 25 cm), with one end capped, were used for the flooded soil column incubations. Four hundred g of dry soil (<1mm) was packed into each PVC column to form a 12-cm soil column. Deionized water was added to the soil columns to achieve saturation and maintain a 4.5-cm layer of overlying water. The flooded soil columns were incubated at 22°C in the dark. Potassium nitrate was amended at 0 or 70 mM (n=2) into the overlying water of the column 1 day after flooding

### **Redox Profile**

The Eh profile in the 0-10 cm layer was measured at a step of 0.5 cm (or 1.0 cm at greater depths) at week 16 for the control soil column and at weeks 8, 12, and 16 for the amended soil column with a platinum electrode as described in Chapter 4.

### **Microbial Activity in Different Redox Zones**

When a stable flat redox profile was formed in the  $\text{NO}_3^-$ -diffusing zone at week 16, soil layers of 0-1, 5-6 and 9-10 cm from the control and  $\text{NO}_3^-$ -diffusing soil columns were sectioned in a glove bag under a  $\text{N}_2$  atmosphere and transferred into 125 ml flasks containing 80 ml deionized water where a soil slurry was maintained with a magnetic stirrer under a  $\text{N}_2$  atmosphere. Four ml of the soil slurry was transferred to a test tube (16

mm x 125 mm) to which had been added 1 ml solution containing: 1) no substrate, 2) 10 mM acetate, or 3) 25 mM glucose. The tube was capped under a N<sub>2</sub> atmosphere and incubated at 22°C with shaking (120 oscillations per min). The incubation time was 4-5 h to also consider the response of fermenting bacteria in the different redox zones. Another set of the test tubes with no substrate addition was aerobically incubated under the above condition. Carbon dioxide and CH<sub>4</sub> concentrations in the headspace of the tube were analyzed (See Chapter 2) for determination of CO<sub>2</sub> and CH<sub>4</sub> production rates expressed as nmol g<sup>-1</sup> h<sup>-1</sup>. The CO<sub>2</sub> production rate was calculated with consideration of CO<sub>2</sub> dissolution in the soil slurry. In addition, pH and NO<sub>3</sub><sup>-</sup> content in the soil slurry prepared from each layer were also measured. Five ml of filtered water (< 0.4 µm) from the soil slurry was analyzed for NO<sub>3</sub><sup>-</sup> content with a NO<sub>3</sub><sup>-</sup>-selective electrode (detection limit 7 x 10<sup>-6</sup> M) (APHA, 1995).

### **Data Analysis**

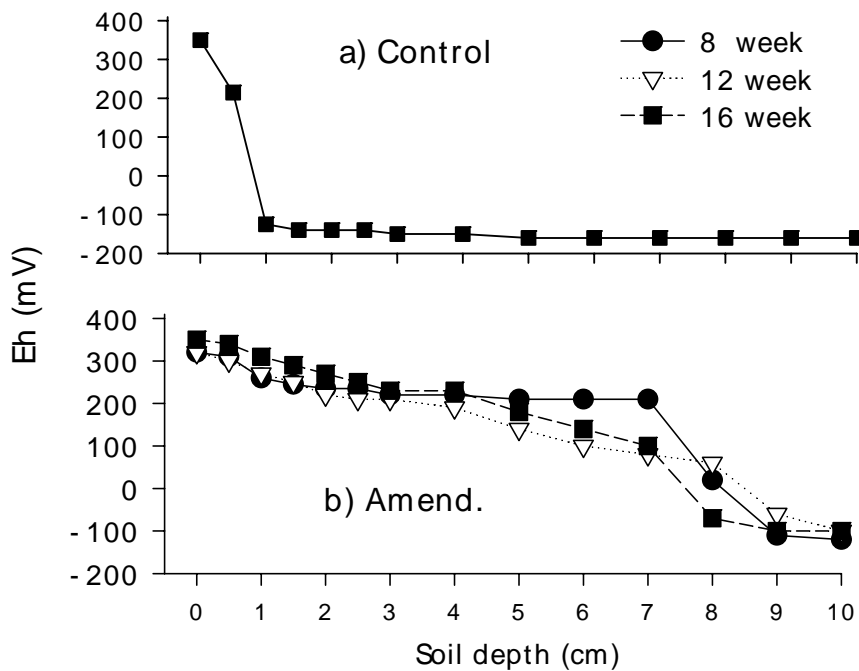
The student t test was used for comparing production of CH<sub>4</sub> and CO<sub>2</sub> as affected by redox zones and substrate addition. Bivariate correlation was performed for establishing the relationship among substrate-induced CO<sub>2</sub> and CH<sub>4</sub> production in all redox zones.

## **Results**

### **Redox Profile**

The Eh profiles were stable in the control and NO<sub>3</sub><sup>-</sup>-diffusing soil columns at week 16 (Fig. 5.1). In the control soil, the redoxcline (redox discontinuity zone from oxic to anoxic) was observed within the 0-2 cm layer and the deeper soil was under highly

reducing conditions. The  $\text{NO}_3^-$  diffusion plume flattened the Eh profile in the 0-7 cm layer with the Eh slope being  $< 50 \text{ mV cm}^{-1}$  and a redox discontinuity zone was formed at the 7- to 8-cm depth. Based on the Eh profile and with consideration of pH and  $\text{NO}_3^-$  availability in the sectioned layers (Table 5.1), the soil layers sectioned from the control and  $\text{NO}_3^-$ -diffusing soil columns were categorized into four redox zones: the 0-1 cm layer of the control soil was the whole-redox-range zone; the 0-1 cm layer of the  $\text{NO}_3^-$ -amended soil was the weakly reducing zone; the 5-6 cm layer of the  $\text{NO}_3^-$ -diffusing soil was the intermediately reducing zone; the 9-10 cm layer of the  $\text{NO}_3^-$ -diffusing soil and the 5-6 and 9-10 cm layers of the control soil were the highly reducing zones.



**Fig. 5.1** Redox profiles of the control and  $\text{NO}_3^-$ -diffusing soil columns

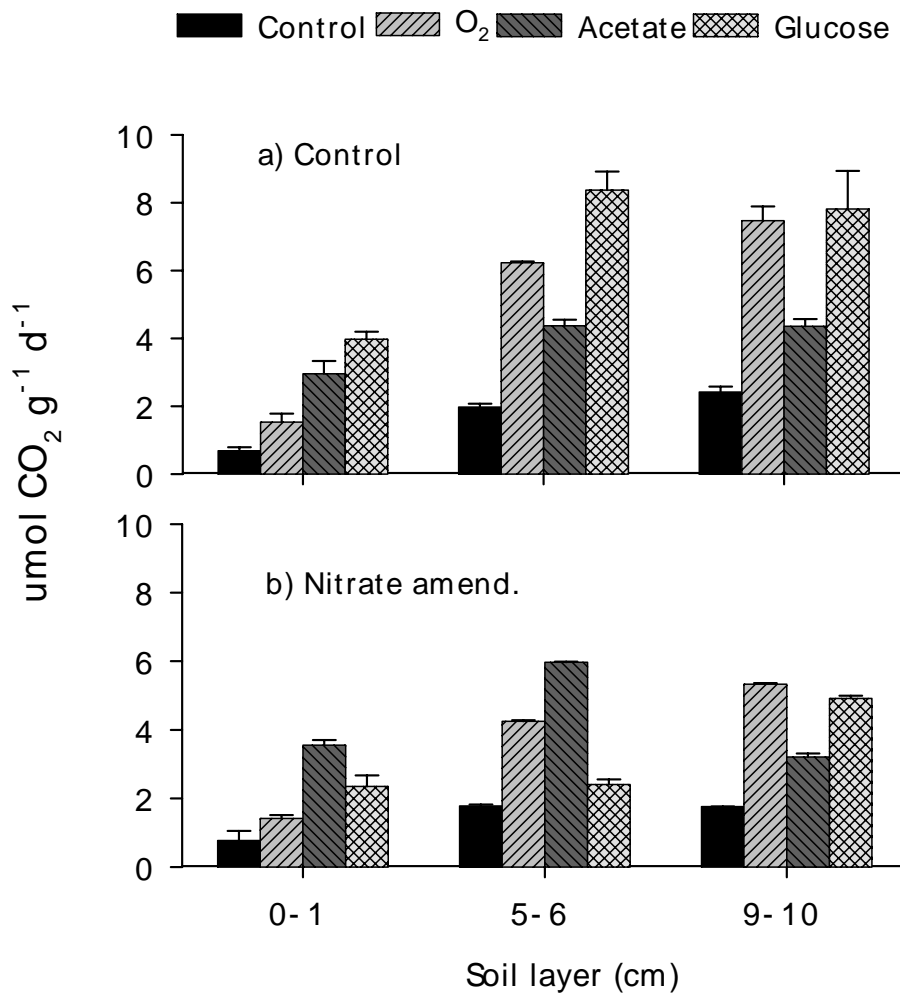
**Table 5.1** Values of pH and NO<sub>3</sub><sup>-</sup> contents in the sectioned soil layers at week 16.

Soil layer (cm)	pH		NO <sub>3</sub> <sup>-</sup> (μmol g <sup>-1</sup> )	
	Control	Amend.	Control	Amend.
0-1	8.0	8.1	ND†	0.34 ± 0.3
5-6	7.7	7.8	-	ND
9-10	7.7	7.7	-	ND

†: Not detectable.

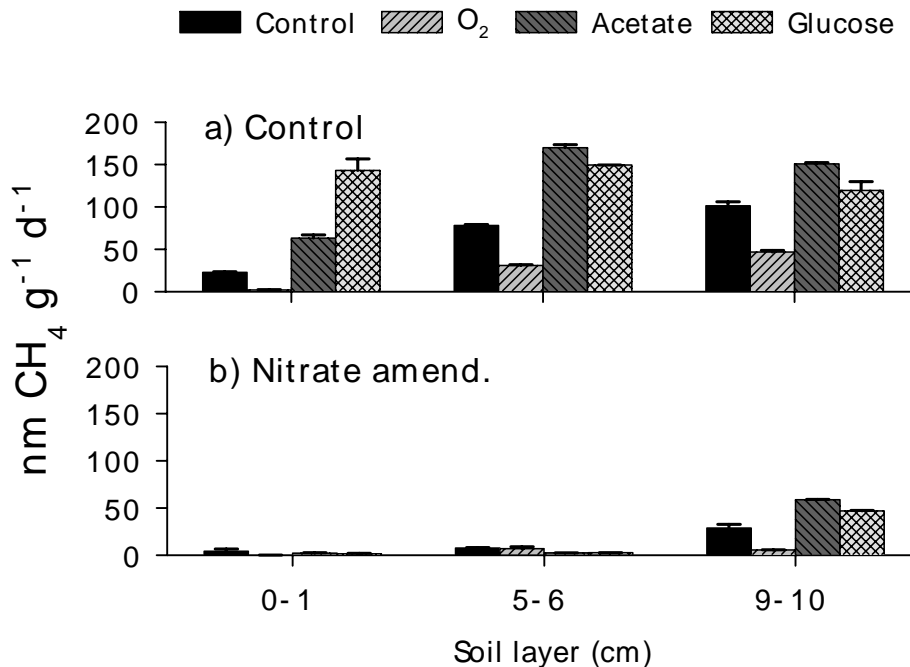
### Carbon Dioxide and Methane Production

After the long-term incubation and without external amendment of carbon sources, CO<sub>2</sub> production in the whole-redox-range zone was slightly lower than in the weakly reducing zone but much lower than in the other redox zones (Fig. 5.2). Methane production was negligible in the weakly and intermediately reducing zones, and relatively small in the whole-redox-range zone, but high in all highly reducing zones (Fig. 5.3). Differences in CH<sub>4</sub> production among the highly reducing zones were also observed, with the rates from the control soil being even higher than that from the NO<sub>3</sub><sup>-</sup>-diffusing soil.



**Fig. 5.2** CO<sub>2</sub> production in different redox zones of the flooded soil. The control, acetate and glucose treatments were anaerobic incubations, while O<sub>2</sub> indicates an aerobic incubation with no carbon substrate addition.





**Fig. 5.3**  $\text{CH}_4$  production in different redox zones of the flooded soil (same explanation as for Fig. 5.2)

### Substrate-Induced Carbon Dioxide and Methane Production

Glucose compared to acetate induced more  $\text{CO}_2$  production in the whole-redox-range and highly reducing zones, and vice versa in intermediately reducing zones ( $p < 0.01$ ) (Fig. 5.2). The amounts of  $\text{CO}_2$  induced by glucose and acetate in the weakly reducing zone were not significantly different. Oxygen induced more  $\text{CO}_2$  production in the intermediate and highly reducing zones than in the weakly reducing and whole-redox-range zones ( $p < 0.05$ ).

Glucose and acetate both significantly stimulated  $\text{CH}_4$  production in the whole-redox-range and highly reducing zones. Acetate compared to glucose induced even more  $\text{CH}_4$  production in the highly reducing zones and the opposite was observed in the

whole-redox-range zone. Methane production was not stimulated by glucose or acetate addition in the weakly and intermediately reducing zones. Oxygen addition significantly suppressed CH<sub>4</sub> production in the highly reducing zones.

### Correlation among Substrate Induced Carbon Dioxide and Methane Production

Correlation analysis showed that glucose- or acetate- induced CO<sub>2</sub> production had a poor correlation while O<sub>2</sub>-induced CO<sub>2</sub> production had a significant correlation with the control CO<sub>2</sub> production (Table 5.2). The correlation was low between acetate- and glucose- induced CO<sub>2</sub> production. Glucose-induced CO<sub>2</sub> production compared to acetate-induced respiration had a better correlation to O<sub>2</sub>- induced respiration.

**Table 5.2** Correlation ( $R^2$ , p) among substrate-induced CO<sub>2</sub> and CH<sub>4</sub> production in all redox zones.

	Control	O <sub>2</sub>	Acetate
CO <sub>2</sub>			
O <sub>2</sub>	0.97 (0.001)		
Acetate	0.53 (0.28)	0.37 (0.47)	
Glucose	0.65 (0.16)	0.78 (0.07)	-0.07 (0.89)
CH <sub>4</sub>			
O <sub>2</sub>	0.97 (0.001)		
Acetate	0.95 (0.003)	0.87 (0.03)	
Glucose	0.63 (0.18)	0.48 (0.34)	0.77 (0.07)

Acetate-induced CH<sub>4</sub> production was significantly correlated to the background CH<sub>4</sub> production. The correlation of glucose-induced CH<sub>4</sub> with the background CH<sub>4</sub> production was relatively low. The inhibition effect of O<sub>2</sub> on CH<sub>4</sub> production was highly correlated to the background CH<sub>4</sub> production.

## Discussion

Glucose- or acetate-induced  $\text{CO}_2$  and  $\text{CH}_4$  production is widely used to estimate microbial activity in anaerobic soils. The applicability of these techniques needs to be evaluated in different redox zones when or where specific anaerobic redox processes dominate. Facultative anaerobes are mainly responsible for denitrification activity and obligate anaerobes are mainly involved in other anaerobic redox processes. Facultative anaerobes use a wide range of substrates, including acetate and glucose, while obligate anaerobes, including methanogens,  $\text{SO}_4^{2-}$  reducers and  $\text{Fe}^{3+}$  reducers, mainly live on acetate-based simple organic carbon and  $\text{H}_2$  derived from fermentation processes as electron donors but cannot directly use or can only use glucose at low efficiencies. Estimates of respiration limited to only glucose- or acetate-induced respiration may not be sufficient to reflect microbial activity in different redox zones as evidenced by this study.

In the intermediate reducing zone dominated by iron-centered redox processes, acetate induced much more  $\text{CO}_2$  production than glucose. This indicated that  $\text{Fe}^{3+}$  reducers could not efficiently use glucose as an electron donor. Also, the relatively small increase of  $\text{CO}_2$  induced by glucose indicated that fermenting activity was weak in this zone, likely due to carbon limitation, and could not rapidly initiate glucose fermentation providing electron donors for  $\text{Fe}^{3+}$  reduction. Such delay in the initiation of fermenting activity was also observed after the depletion of  $\text{NO}_3^-$  in the slurry experiment (Chapter 2). In contrast, in the highly reducing zones, glucose induced much more  $\text{CO}_2$  production

and similar CH<sub>4</sub> production compared to acetate, indicating glucose fermentation was rapidly initiated and provided sufficient substrates for methanogens. In the weakly reducing and whole-redox range zones, the responses of anaerobes to acetate and glucose were not significantly different, due to the important role of facultative anaerobes in the two zones. While microbial activity in the two zones was low due to carbon limitation under O<sub>2</sub> diffusion, the relative increases in CO<sub>2</sub> production by acetate and glucose additions were comparable to or even higher than those in the other redox zones.

Thus, both glucose and acetate had advantages and disadvantages for estimation of microbial activity in the different redox zones. Acetate-induced respiration did not reflect fermenting activity, but was better in estimating microbial activity in the intermediately reducing zone. Overall, glucose-induced respiration was better in the other redox zones, as supported by its better correlation to O<sub>2</sub>-induced respiration, which indicated the availability of carbon sources and significantly correlated to the background anaerobic respiration. Acetate- and glucose- induced respiration may be both measured to better account for the effect of redox conditions, or more specifically, the relative importance of specific redox processes, on microbial activity in anaerobic soils.

## **Conclusions**

Both glucose- and acetate-induced CO<sub>2</sub> and CH<sub>4</sub> production had disadvantages and advantages for microbial activity estimation in different redox zones when specific anaerobic redox processes dominated. Glucose induced respiration was as effective as acetate to reflect the activity of obligate anaerobes in the highly reducing zone, but was

not in the intermediately reducing zone where fermenting activity could not be quickly initiated after glucose addition. While acetate-induced respiration did not reflect fermenting activity in the highly reducing zone, it did not significantly differ from glucose-induced respiration in the weakly reducing and whole-redox-range zones where facultative anaerobes dominated.

Acetate appeared to better reflect microbial activity in the intermediately reducing zone where iron-centered intermediate redox processes dominated while glucose was better overall in the other redox zones. To better account for the effect of redox condition on microbial activity, respiration induced by acetate and glucose may be both used for microbial activity assessment in anaerobic soils under different redox conditions.

## **CHAPTER 6**

### **BIOELECTROCHEMICAL NITRATE REMOVAL FROM WATER USING FLOODED SOIL AS A REDUCTANT SOURCE**

#### **Introduction**

Huge amounts of energy sources other than fossil fuels exist at low densities in anaerobic environments but difficult to exploit (Reimers et al., 2001; Bond et al., 2002). Redox gradients are established in anaerobic environments where a difference in redox potential typically ranging around 0.8 V can be found within a distance as short as a few centimeters centering at oxic and anaerobic interfaces. This is sufficient to support electricity generation through a fuel cell process (Reimers et al., 2001; Bond et al., 2002). Many groups of microorganisms and electron shuttling substances are involved in the process of bioelectricity generation (Reimers et al., 2001; Bond et al., 2002; Rabaey et al., 2003; Chaudhuri and Lovley, 2003; Gregory et al., 2004; Liu et al., 2004). The microbially harvested power normally ranges from 10 to 100 mW m<sup>-2</sup> (Liu et al., 2004) and is able to drive low-power instruments in marine environments (Reimers et al., 2001; Bond et al., 2002). Difficulties exist in methods to collect, concentrate and store this bioelectricity for practical applications.

Nevertheless, since the internal resistance of a biobattery is relatively high, such relatively low bioelectricity may not be harvested through an external load but generated directly for bioelectrochemical pollutant removal. During bioelectricity generation from wastewater, organic matter is bioelectrochemically oxidized by O<sub>2</sub> (Liu et al., 2004).

Oxidized pollutants such as  $\text{NO}_3^-$  can be bioelectrochemically reduced when  $\text{O}_2$  is not available (Gregory et al., 2004). Nitrate as a major pollutant in drinking water can be removed with physico/chemical and biological processes (Kapoor and Viraraghavan, 1997). While a biological process is cost-effective compared to expensive physico/chemical processes such as reverse osmosis and ion-exchange, a readily reducible carbon substrate such as ethanol normally needs to be added to the water being treated (Fuchs et al., 1997). With the biobattery approach, a wide range of organic carbon sources (e.g. agricultural biomass) may be applicable for  $\text{NO}_3^-$  removal without direct contact with the water. The use of organic matter as an energy source for microbes with the biobattery approach also makes it different from electrochemical/bioelectrochemical  $\text{NO}_3^-$  removal by an electric current field generated by typical electricity generating sources (Feleke and Sakakibara, 2001; Zaveri and Flora, 2002; Beschkov et al., 2004).

Relatively little research has been done on the use of microbially mediated reductants for bioelectrochemical  $\text{NO}_3^-$  removal. A recent report shows that an electrode poised at -300 mV (against the standard  $\text{H}_2$  electrode) with a potentiostat as the source of electron donors reduces  $\text{NO}_3^-$  with nitrite being the dominant end product (Gregory et al., 2004). While wastewaters and soils/sediments may develop low redox potentials close to -300 mV, bioelectrochemical reduction of  $\text{NO}_3^-$  by microbially mediated reductants from these environments may differ from that by a poised electrode.

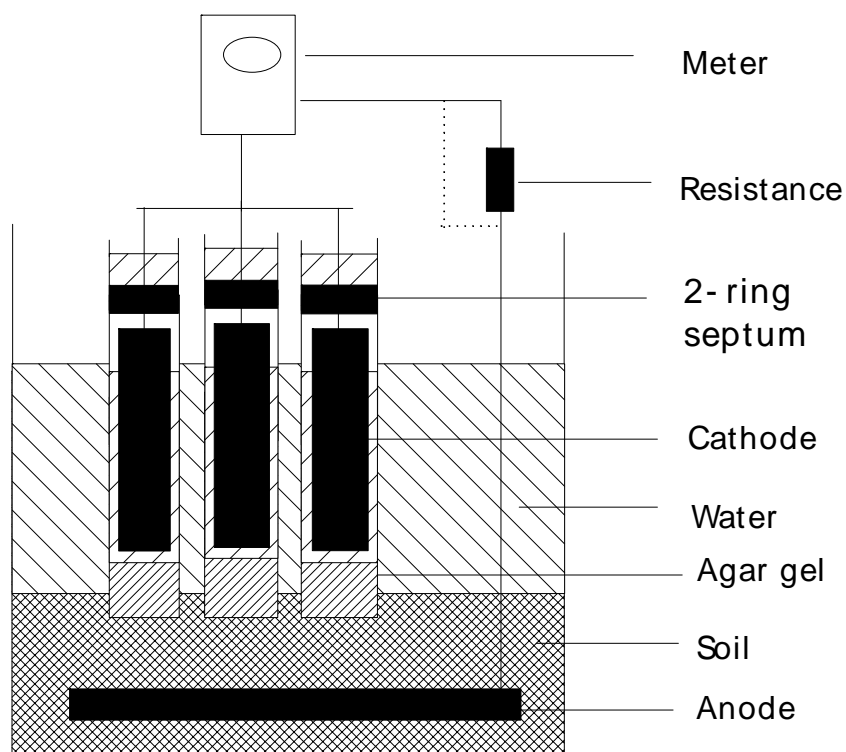
In this study, the ability of a flooded soil as a source of reductants to bioelectrochemically reduce  $\text{NO}_3^-$  in carbon-limiting water was evaluated. The role of

N<sub>2</sub>O as an intermediate product of NO<sub>3</sub><sup>-</sup> reduction in bioelectricity generation was also studied.

## Materials and Methods

### Microbial Fuel Cell Set Up

The microbial fuel cell using a flooded soil as a reductant source is shown in Fig. 6.1.



**Fig. 6.1** A microbial fuel cell using a flooded soil as a reductant source

The electrode placed in the reducing soil layer is the anode and the cathode is placed in the overlying water. The flooded soil, already under a 2-month flooding preincubation, was mixed with 0.05% glucose and put into a plastic vessel (25 cm x 30 x 18 cm) to form a 6-cm soil layer. For properties of the soil, see the Louisiana soil from Table 2.1 in Chapter 2. A graphite plate (15 mm x 100 mm x 150 mm), coarsened with sand paper, electrically connected to a copper wire with silver conductive epoxy covered with



nonconductive epoxy (Reimers et al., 2001), was placed at the 4-cm depth of the flooded soil to serve as the anode.

When the soil had developed highly reducing conditions (e.g. redox potentials below the 1-cm depth  $< -200$  mV) after 1 more week of incubation at 25°C, a 140-ml plastic syringe (inner diameter 3.7 cm) was placed at the 1-cm depth of the flooded soil to serve as the cathode chamber after modification. The tip near the body of the syringe was cut to get a 2-cm diameter hole. A rubber stopper temporally sealed the hole while hot agar gel (4%) was poured into the syringe to form a 3-cm gel layer upon cooling, and then the stopper was removed. The gel layer was used as the separator between the cathode chamber and the flooded soil. Fifty ml of water was poured on top of the agar gel in the syringe. One piece of carbon paper (170  $\mu\text{m}$  x 3.5 cm x 6 cm, AvCarb-p50, Ballard Power System) as a cathode was put into the syringe and was partly submerged (3.5 cm x 4 cm). The depth of overlying water in the vessel was adjusted to be same as that in the syringe during electricity generation as described below.

### **Electricity Generation Coupled to Oxygen Reduction**

The cathode chamber was filled with 50 ml of tap water and kept aerobic. Three cathodes were connected in parallel and then were connected to the anode with the option of going through a 465-ohm external resistor or bypassing this resistor during electricity generation. The ratio of the anode area relative to the total effective cathode area was 3.6 to facilitate electron transfer by the anode from the static flooded soil. The electric current was measured once per minute for 1 day using a digital multimeter (RadioShack

22-812) serially connected between the anode and cathodes and was recorded by a computer using Meter View 1.0 through a RS232 port.

### **Electricity Generation Coupled to Nitrate Reduction**

The cathode chamber was filled with 50 ml solution containing 0.8% sodium chloride and 5 mM potassium nitrate and inoculated with soil at 0.2 or 1.0% (from the top aerobic layer of the flooded soil without glucose addition) under a N<sub>2</sub> atmosphere and capped with a two-ring septum leaving a headspace of 47 ml. The septum was covered with a 1-cm layer of overlying water to ensure anaerobic conditions in the cathode chamber. Then the cathode chamber was anaerobically incubated for 3 d with or without electricity generation. No external resistance was used during electricity generation. A control with no soil inoculation and no current generation was included to account for NO<sub>3</sub><sup>-</sup> change due to diffusion into the gel layer.

Based on the current variation, gas samples (2 ml) were taken from the headspace of the cathode chamber at hours 20 and 60 at the low inoculation rate and at hours 20 and 40 at the high inoculation rate and analyzed with a Tremetrics 9001 gas chromatograph using an electron capture detector (ECD) for determining N<sub>2</sub>O concentrations. At the end of the experiment, water samples from the cathode chambers were taken, filtered (<0.4 µm), and analyzed for NO<sub>3</sub><sup>-</sup> content by a NO<sub>3</sub><sup>-</sup> selective ion electrode (APHA, 1995). The electrons transferred into the cathode chamber through current flow were calculated according to 1 coulomb = 1 ampere x 1 second and 1 mole = 96,485 coulombs.

## **Electricity Generation Coupled to Nitrous Oxide Reduction**

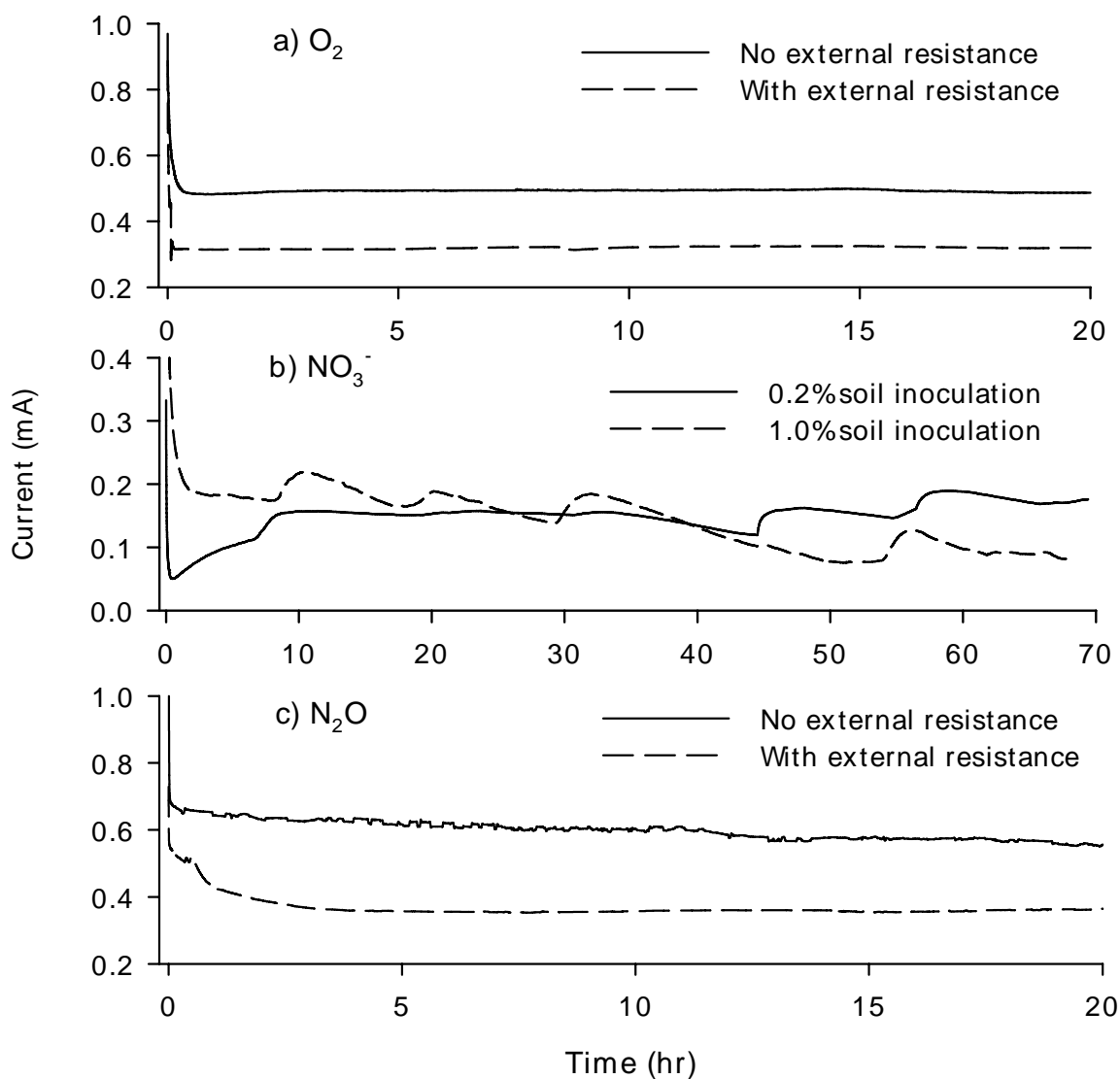
The procedure was the same as for “electricity generation coupled to  $\text{NO}_3^-$  reduction” except that the cathode chamber was filled with 50 ml of solution containing 20 mM potassium nitrate and 5 mM glucose and inoculated with 4.0% soil and that the headspace of the cathode chamber was amended with acetylene at 20% for inhibition of enzymatic  $\text{N}_2\text{O}$  reduction to  $\text{N}_2$  (Tiedje, 1982). Half an hour after the addition of acetylene, the current was measured for 1 d with and without a 465-ohm external resistor in the circuit.

## **Results**

### **Electricity Generation**

Electricity was consistently generated when the cathode chamber was kept aerobic (Fig. 6.2a). The current was  $32.9 \text{ mA m}^{-2}$  with no external resistance and  $21.4 \text{ mA m}^{-2}$  with a 465-ohm external resistance based on the anode area. The power harvested through the external resistance was  $3.2 \text{ mW m}^{-2}$  based on the anode area, but  $11.4 \text{ mW m}^{-2}$  based on the cathode area.

The current coupling to  $\text{NO}_3^-$  reduction was variable with different patterns at the two inoculation rates and averaged  $\sim 70.0\%$  lower than when coupled to  $\text{O}_2$  reduction (Fig. 6.2b). The current at the high inoculation rate was high with repeated peaks in the first 40 hours and became small thereafter. The current at the low inoculation rate decreased rapidly soon after the start of electricity generation, gradually increased to  $\sim 0.16 \text{ mA}$  at hour 10, and then was stable around this level until hour 35 and became variable with two peaks thereafter.



**Fig. 6.2** Current generation coupled to O<sub>2</sub> reduction (a), NO<sub>3</sub><sup>-</sup> reduction (b), and N<sub>2</sub>O reduction (c) with or without a 465-ohm external resistance. No external resistance was used when nitrate was the oxidant.

Nitrous oxide was detected ( $> 2.5 \mu\text{L L}^{-1}$ ) in the headspace of the cathode chambers with the higher concentration of N<sub>2</sub>O corresponding to the higher current at each inoculation rate (Table 6.1). When N<sub>2</sub>O accumulated under the inhibition by acetylene of

the enzymatic reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ , the current generated was stable and was 21% higher than that coupled to  $\text{O}_2$  reduction with no external resistance and 15% higher with a 465-ohm external resistance (Fig. 6.2c).

**Table 6.1** Nitrous oxide concentrations (mean  $\pm$  SE, n=3) in the headspace of the cathode chambers under current generation.

Inoculation	Hour	$\text{N}_2\text{O}$ ( $\mu\text{ L L}^{-1}$ )	Current (mA)
0.2%	20	$2.68 \pm 0.87$	0.15
	60	$3.92 \pm 0.68$	0.19
1.0%	20	$3.44 \pm 0.73$	0.18
	40	$2.98 \pm 0.54$	0.12

### Nitrate Removal under Electricity Generation

Using the decrease in  $\text{NO}_3^-$  content in the non-inoculation treatment to account for the effect of  $\text{NO}_3^-$  diffusion in the inoculation treatments, the  $\text{NO}_3^-$  removal under electricity generation was faster than under no electricity generation at each inoculation rate (Table 6.2). With or without electricity generation, the  $\text{NO}_3^-$  removal at the high inoculation rate was >3 times faster than that at the low inoculation rate. The ratios of electrons transferred through current flow to the amount of current flow-induced  $\text{NO}_3^-$  removal were 2.2 at the high inoculation rate and 6.1 at the low inoculation rate.

**Table 6.2** Nitrate contents (mean  $\pm$  SE, n=3) in the cathode chambers. The chambers were amended with 5 mM potassium nitrate and anaerobically incubated for 3 days with or without current generation.

Inoculation	Treatment	Decrease in NO <sub>3</sub> <sup>-</sup> content (mM)
-	Control	1.1 $\pm$ 0.1
0.2%	No current	1.3 $\pm$ 0.0
	With current	1.7 $\pm$ 0.1
1.0%	No current	2.1 $\pm$ 0.2
	With current	3.3 $\pm$ 0.1

## Discussion

### Electricity Generation

The ability of the static flooded soil as a source of reductants to support bioelectricity generation coupled to O<sub>2</sub> reduction was low based on the anode area, but was within the normal reported range of 10-100 mW m<sup>-2</sup> based on the cathode chamber (Liu et al., 2004). The use of a 3-cm layer of agar gel instead of a proton exchange membrane (e.g., Nafion) as the separator for the cathode chamber and the reducing soil increased the internal resistance between them. A Nafion membrane would not function well under direct contact with bulk soil. The static states of the reducing soil and the cathode chamber may also lower the ability of bioelectrochemically active anaerobes to transfer electrons on the anode and cathode surfaces (Bond et al., 2002; Gregory et al., 2004).

The lower currents coupled to NO<sub>3</sub><sup>-</sup> reduction compared to that coupled to O<sub>2</sub>

reduction showed that  $\text{NO}_3^-$  reduction was not as efficient as  $\text{O}_2$  reduction in accepting electrons from current flow. Further, the current coupled to  $\text{NO}_3^-$  reduction was variable. The anode was not expected to be a factor for the current variations since it constantly transferred electrons when the cathode chamber was aerobic. Such current variations would be largely attributed to the variations in  $\text{NO}_3^-$  reduction and  $\text{N}_2\text{O}$  accumulation in the cathode chambers affected by the inoculation rate. The electrons transferred through current flow directly stimulated  $\text{NO}_3^-$  reduction to nitrite (Gregory et al., 2004), but more likely promoted the reduction of  $\text{N}_2\text{O}$  and  $\text{NO}$  due to their higher redox potentials (Handbook of Chemistry and Physics, 1991) when they were accumulated in the cathode chambers under carbon limitation (Benckiser et al., 1996). According to the concentrations of  $\text{N}_2\text{O}$  in the headspace,  $\text{N}_2\text{O}$  dissolved in the solution was even sufficient as the oxidant to accept the electrons transferred through current flow. Nevertheless, the slower reduction of  $\text{NO}_3^-$  at the low inoculation rate due to a greater extent of carbon limitation and a lower density of microorganisms would delay the accumulation of  $\text{N}_2\text{O}$  and  $\text{NO}$  resulting in the lower currents in the first 10 hours and vice versa at the high inoculation rate, and, the faster removal of  $\text{NO}_3^-$  at the high inoculation rate would lower the accumulation of  $\text{N}_2\text{O}$  and  $\text{NO}$  later resulting in the lower currents in the late hours.

The importance of  $\text{N}_2\text{O}$  in electricity generation was directly reflected by the electricity generation coupled to  $\text{N}_2\text{O}$  reduction (Fig. 2c). Sufficient glucose was added into the cathode chamber to stimulate  $\text{NO}_3^-$  reduction but  $\text{N}_2\text{O}$  accumulated due to the

inhibition of acetylene on the enzymatic reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  (Tedje, 1982). However,  $\text{N}_2\text{O}$  is a strong oxidant comparable to  $\text{O}_2$  but is much more soluble (Handbook of Chemistry and Physics, 1991). Nitrous oxide reduction, compared to  $\text{O}_2$  reduction, resulted in generation of the even higher currents.

### **Bioelectrochemical Nitrate Removal**

An imbalance in electron transfer between current generation and  $\text{NO}_3^-$  removal was observed. The ratio of the amount of electrons transferred through current flow to that of  $\text{NO}_3^-$  removed due to current generation was 6.1 at the low inoculation rate, close to the ratio 5 for reduction of  $\text{NO}_3^-$  to  $\text{N}_2$  and this ratio became 2.2 at the high inoculation, close to the ratio 2 for reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  or  $\text{NO}$  to  $\text{N}_2$ . In other words,  $\text{NO}_3^-$  removal under current generation was more efficient at the high inoculation rate. Such inconsistencies have been reported elsewhere (Gregory et al., 2004). Due to a greater extent of carbon limitation and a lower density of microorganisms,  $\text{NO}_3^-$  removal under current generation at the low inoculation rate was largely associated with current flow (Gregory et al., 2004). In contrast,  $\text{NO}_3^-$  removal at the high inoculation rate under no current generation was even higher than that at the low inoculation rate under current generation. The bioelectrochemical reduction of  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$  at the high inoculation rate likely lowered the extent of carbon limitation in the biological  $\text{NO}_3^-$  reduction, e.g. the step from  $\text{N}_2\text{O}$  to  $\text{N}_2$ , to stimulate biological reduction overall. The high inoculation rate also favored the cloning of bioelectrochemically active microorganisms on the cathode (Gregory et al., 2004) contributing to the more efficient  $\text{NO}_3^-$  removal.



The function of  $\text{NO}_3^-$  as an oxidant in electricity generation provides the promise of bioelectrochemical  $\text{NO}_3^-$  removal from carbon-limiting environments such as pretreated drinking waters. The rates of bioelectrochemical  $\text{NO}_3^-$  removal in this study are within the low part of the range of denitrification in natural environments (Davidsson and Stahl, 2000; Hunter and Faulkner, 2001) but are considerable for  $\text{NO}_3^-$  removal from drinking water. The efficiency of  $\text{NO}_3^-$  removal by the biobattery approach may be further increased when new proton exchange media functioning well in contact with bulk soils/sediments become available to increase bioelectricity generation. The biobattery approach may be combined with the biological approach to lower the requirement of readily reducible substrates for  $\text{NO}_3^-$  removal from drinking water.

### **Conclusions**

Through increasing the anode area relative to the cathode area, microbially mediated reductants from the static flooded soil were able to support current generation coupled to the reduction of  $\text{O}_2$  dissolved in the overlying water. The current was lowered when  $\text{NO}_3^-$  was substituted for  $\text{O}_2$ , but  $\text{NO}_3^-$  removal was significantly stimulated compared to that under no current generation suggesting the applicability of a wide range of organic carbon sources for bioelectrochemical  $\text{NO}_3^-$  removal from drinking water. The current generation with  $\text{NO}_3^-$  as the oxidant not only coupled the reduction of  $\text{NO}_3^-$  to nitrite but also the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ . The current varied with microbial  $\text{NO}_3^-$  reduction and  $\text{N}_2\text{O}$  accumulation affected by the inoculation rate. When  $\text{N}_2\text{O}$  accumulation was high

under the inhibition of acetylene on the enzymatic reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ , the current generated was even higher than that coupled to  $\text{O}_2$  reduction.

## **CHAPTER 7**

### **EFFECTS OF FERRIC IRON AMENDMENT ON NITRATE PERCOLATION AND EMISSIONS OF NITROUS OXIDE AND METHANE IN A RICE SOIL**

#### **Introduction**

Iron redox cycling plays a key role in organic carbon decomposition, metal mobilization and immobilization, nutrient release, and biogeochemistry of  $\text{NO}_3^-$  and  $\text{CH}_4$  in non-sulfidogenic anaerobic environments (Lovley, 1991; Lovley et al., 1994; Roden and Wetzel, 1996; Yao et al., 1999; Senn et al., 2002).

There is increasing interest in the role of iron redox cycling in  $\text{CH}_4$  biogeochemistry in rice fields (Roden and Wetzel, 1996; Frenzel et al., 1999; Matthews et al., 2000). Rice fields are a major biogenic source of  $\text{CH}_4$ , an atmospheric trace gas second only to  $\text{CO}_2$  in its contribution to global warming (IPCC, 2001). Ferric iron generally constitutes the major part of the alternative electron acceptor (AEA) pool in rice soils (Yao et al., 1999). Microbial  $\text{Fe}^{3+}$  reduction suppresses  $\text{CH}_4$  production in flooded soils, especially under substrate-limiting conditions (Achthich et al., 1995). Therefore, increasing  $\text{Fe}^{3+}$  reduction intensity, e.g. through  $\text{Fe}^{3+}$  amendment, reduces  $\text{CH}_4$  emission from rice fields (Watanabe and Kimura, 1999; Jäkel and Schnell, 2000; Furukawa and Inubushi, 2002).

More importantly, iron redox cycling affects  $\text{CH}_4$  dynamics in rice fields through  $\text{Fe}^{3+}$  regeneration. Ferric iron is the dominant AEA capable of regeneration in rice soils. Ferric iron regeneration at the oxygenated soil-water interface and in the oxidized rhizosphere is important in controlling root exudation and  $\text{CH}_4$  dynamics (Frenzel et al.,

1999; King and Garey, 1999; Ratering and Schnell, 2000). Ferric iron regeneration stimulated by drainage has been suggested to be important in drainage-induced CH<sub>4</sub> mitigation (Sigren et al., 1997; Ratering and Conrad, 1998). Nevertheless, while drainage is most effective and promising in reducing CH<sub>4</sub> emission (Yagi et al., 1997), few experimental evaluations are available on the role of Fe<sup>3+</sup> regeneration in drainage-induced CH<sub>4</sub> mitigation. Such evaluations are especially needed before adoption of a short-term drainage-based recommendation for CH<sub>4</sub> mitigation while lowering the risks of reducing rice yield and increasing the emission of another very important greenhouse gas, N<sub>2</sub>O (Yagi et al., 1997; Henckel and Conrad, 1998; Cai et al., 1999).

Iron redox cycling also affects NO<sub>3</sub><sup>-</sup> biogeochemistry in anaerobic environments. Anaerobic oxidation of Fe<sup>2+</sup> may be coupled to NO<sub>3</sub><sup>-</sup> reduction (Benz et al., 1998; Nielsen et al., 1998; Senn et al., 2002) and promote N<sub>2</sub>O formation (Brons et al., 1991; van Cleemput et al., 1996). Aerobic Fe<sup>2+</sup> reoxidation at the aerobic/anaerobic interfaces of wetland soils may regulate O<sub>2</sub> availability (van Bodegom et al., 2001; Neubauer et al., 2002) and thereby affect NO<sub>3</sub><sup>-</sup> reaction dynamics and N<sub>2</sub>O formation. Fate of NO<sub>3</sub><sup>-</sup> in wetland soils may also be altered by intensified reduction of Fe<sup>3+</sup>-centered AEAs in the deep soil layer (Chapter 4).

Here we present a study on the effect of intensity of Fe<sup>3+</sup> reduction on NO<sub>3</sub><sup>-</sup> percolation, N<sub>2</sub>O and CH<sub>4</sub> fluxes in a potted rice soil. The effect of Fe<sup>3+</sup> reduction and regeneration intensity on N<sub>2</sub>O and CH<sub>4</sub> fluxes was evaluated in control and ferrihydrite-amended rice pots before and after short-term drainage.

## Materials and Methods

### Experimental Setup

A Louisiana rice soil was used in the experiment. The soil sample was collected from the field top layer (0-20 cm), air-dried, stored at room temperature (20 °C), and sieved through 4-mm mesh. See Table 2.1 in Chapter 2 for the soil properties.

The pot experiment was carried out outside in early June, 2003. The pot was placed with or without a 3-cm layer of quartz sand and glass wool at the bottom. Three kilograms of soil (dw) was mixed with 1000 ml fertilizer solution in a bucket, transferred into the pot, and stabilized under flooded conditions for 2 days. The fertilizer solution contained 0.98 g  $\text{KH}_2\text{PO}_4$  and 0.48 g urea with or without amendment of 10.50 g ferrihydrite-Fe. The ferrihydrite-containing fertilizer solution was prepared by mixing ferrihydrite suspension with the fertilizers just before mixing with the soil. The addition rate of Fe was equivalent to  $63 \mu\text{mol g}^{-1}$ , a rate comparable to indigenous reducible iron content in the soil. Two days after flooding, each pot was planted with three 21-day-old rice seedlings (*Oryza sativa* L.). The pots were watered if needed to maintain a 2-4 cm layer of overlying water in the first month after flooding, and 5-7 cm afterwards. Weeds in the pots were manually removed weekly. Urea (0.32 g) was applied twice as top dressing on days 37 and 67.

The pots with quartz sand and glass wool placed at the bottom were used for the experiment on  $\text{NO}_3^-$  percolation (see below). The other pots were used for the experiments on  $\text{N}_2\text{O}$  and  $\text{CH}_4$  emission. These pots were drained by removal of overlying water either once (on Day 55 after flooding for 2 days) or twice (on days 55 and 85, both for 2 days).

The selections of drainage dates were based on the observance of large increases of CH<sub>4</sub> emissions from the pots with consideration of rice growth stage. All the pots were drained on day 114 for harvest on day 127. Thus, there were 4 combinations of ferrihydrite amendment and drainage treatments to study emissions of CH<sub>4</sub> and N<sub>2</sub>O, and each combination was done in triplicate.

### **Nitrate Percolation**

From day 3 to 22 after flooding, 110 ml water was collected each day from the bottom of the pot (n=3) with a 140-ml syringe, equivalent to a percolation rate of 4.0 mm d<sup>-1</sup>. Percolates taken on days 3, 6, 16 and 22 were filtered (<0.4 µm) for NO<sub>3</sub><sup>-</sup> content determination by a NO<sub>3</sub><sup>-</sup> selective electrode (APHA, 1995).

### **Nitrous Oxide and Methane Emissions**

The closed chamber method was used for measurement of N<sub>2</sub>O and CH<sub>4</sub> emissions from the pots. Transparent cylindrical plastic chambers (inner diameter 12.5 cm) were used during different rice-growing periods. Each chamber was installed with a small battery-operated fan for gas mixing and a rubber septum for gas sampling. An equilateral triangle-shape wood frame was permanently fixed onto the inner sidewall of the pot just above the soil-water interface to be used as the base to hold the chamber during gas sampling. The air in the chamber was mixed by the fan and gas samples were taken by a 10-ml air-tight syringe at 0, 10, 20 and 30 min after positioning the chamber onto the base. Gas sampling was carried out between 9:30 and 11:00 in the morning on days 4, 6 and 10 after flooding, and weekly or biweekly afterwards. Methane emissions from the pots were

measured until day 113. Nitrous oxide emissions were measured during the first 22 days, and 1 day after the reflooding of the drained soil in the mid- and late-seasons. Within one day after sampling, gas samples were analyzed with a Tremetrics 9001 gas chromatograph using an electron capture detector (ECD) and a flame ionization detector (FID) for determination of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  concentrations, respectively. The  $\text{N}_2\text{O}$  and  $\text{CH}_4$  fluxes were determined as the amounts of gas accumulated versus time over a unit of surface area. The cumulative  $\text{CH}_4$  emission from the pot was computed as the sum of the production rate on each sampling date multiplied by the time interval which was calculated from the midpoints of neighboring sampling dates.

### **Relationship between Ferric Iron Regeneration and Methane Emission**

Reliable estimates of  $\text{Fe}^{3+}$  reduction or regeneration in the potted soil before and after drainage was not possible due to soil heterogeneity (Ratering and Schnell, 2000). The effect of ferrihydrite amendment on reducing  $\text{CH}_4$  emission before the midseason drainage was not considered since  $\text{CH}_4$  emission from each treatment in this period was similarly small and accounted for <10% of the cumulative emission during the 113-day measurement. Ferric iron regeneration during flooded periods was assumed to be largely controlled by  $\text{O}_2$  diffusion but not by the availability of reoxidizable  $\text{Fe}^{2+}$  and hence not by  $\text{Fe}^{3+}$  amendment. Then, regression analysis was conducted between potential drainage-induced  $\text{Fe}^{3+}$  regeneration and cumulative  $\text{CH}_4$  emission during the whole measurement period. The potential  $\text{Fe}^{3+}$  regeneration during drainage was calculated as 100% regeneration of reducible iron in the soil. The amount of the reducible iron was

estimated according to the reducibility of indigenous soil iron and/or ferrihydrite from the slurry experiment (see Table 2.2 in Chapter 2).

### **Rice Yield and Aboveground Biomass**

After harvest, rice yield per pot was determined by weighing. Aboveground biomass (excluding rice seeds) per pot was determined by weighing after drying at 105 °C for 24 h.

### **Statistical Analysis**

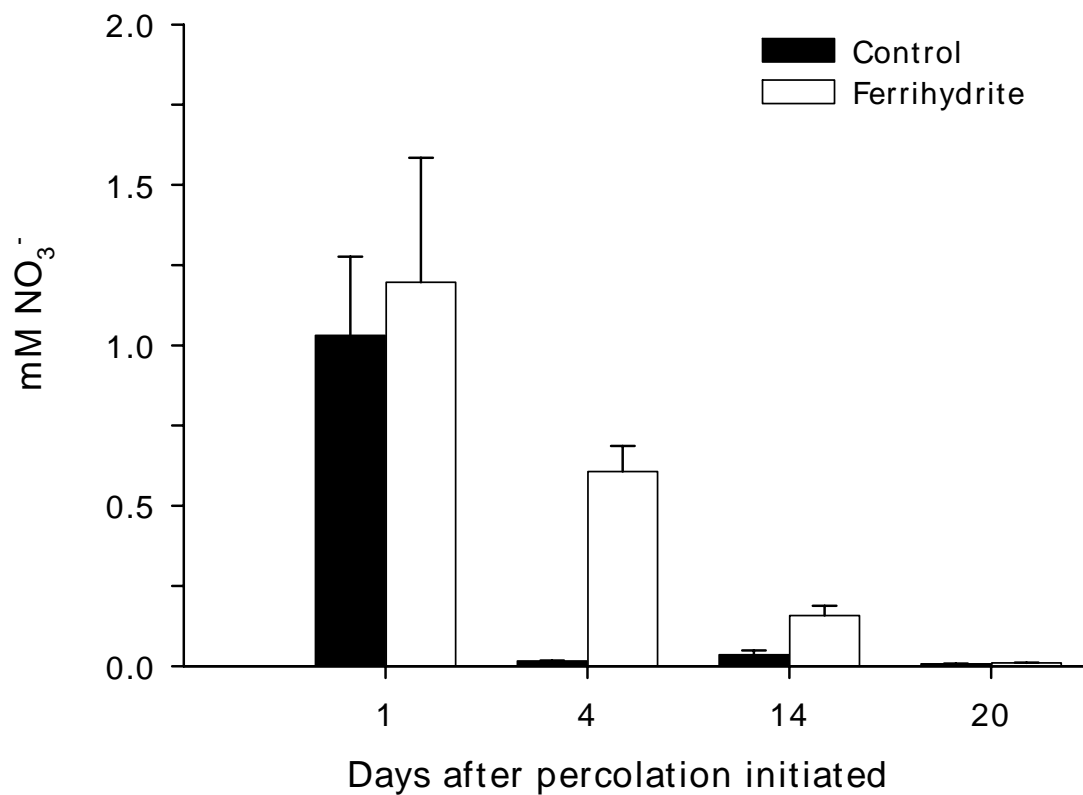
Statistical analysis was conducted with SAS (SAS Institute Inc., 1999-2001). Differences in  $\text{NO}_3^-$  percolation, aboveground biomass and rice yield between the treatments were evaluated with Student t test, and seasonal variation of  $\text{CH}_4$  emission between the treatments was evaluated with one-way analysis of variance (ANOVA). A linear regression was conducted between potential of drainage-induced  $\text{Fe}^{3+}$  regeneration and cumulative  $\text{CH}_4$  emission in the potted soil. The statistical level of significance was chosen at  $\alpha = 0.05$  for all statistical analysis.

## **Results**

### **Nitrate Percolation**

Nitrate percolation was detected at  $>1.0 \text{ mM NO}_3^-$  in both treatments on day 1 after percolation (Fig. 7.1). Nitrate percolation was negligible in the control soil by day 4 but continued until between day 14 and 20 in the amended soil.

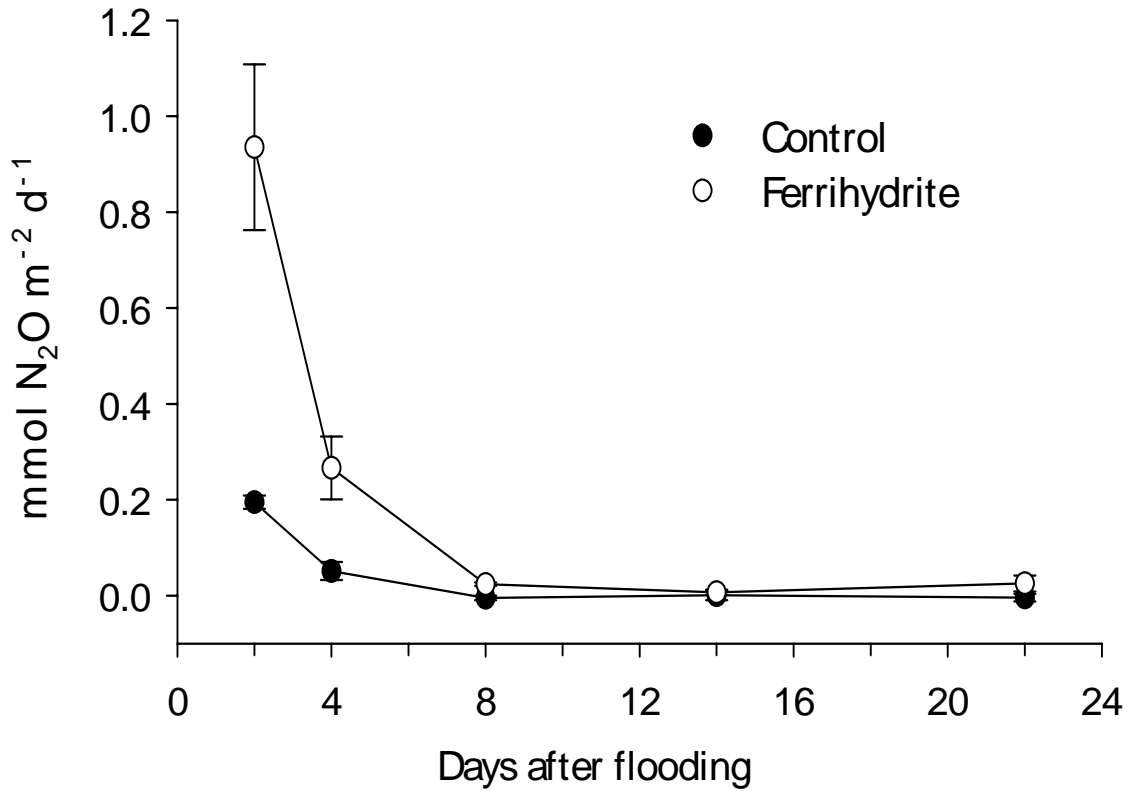




**Fig. 7.1** Nitrate percolation (mean  $\pm$  SE, n=3) from the potted soil affected by ferrihydrite amendment. Percolation started at 4.0 mm d<sup>-1</sup> 2 days after flooding.

### Nitrous Oxide and Methane Emissions

Significant N<sub>2</sub>O fluxes were observed, which were increased by ferrihydrite amendment at early phase of flooding (Fig. 7.2). Nitrous oxide fluxes were small in both treatments after flooding for 10 days. No significant N<sub>2</sub>O fluxes were observed 1 day after reflooding of the drained pots in the mid- and late-seasons.

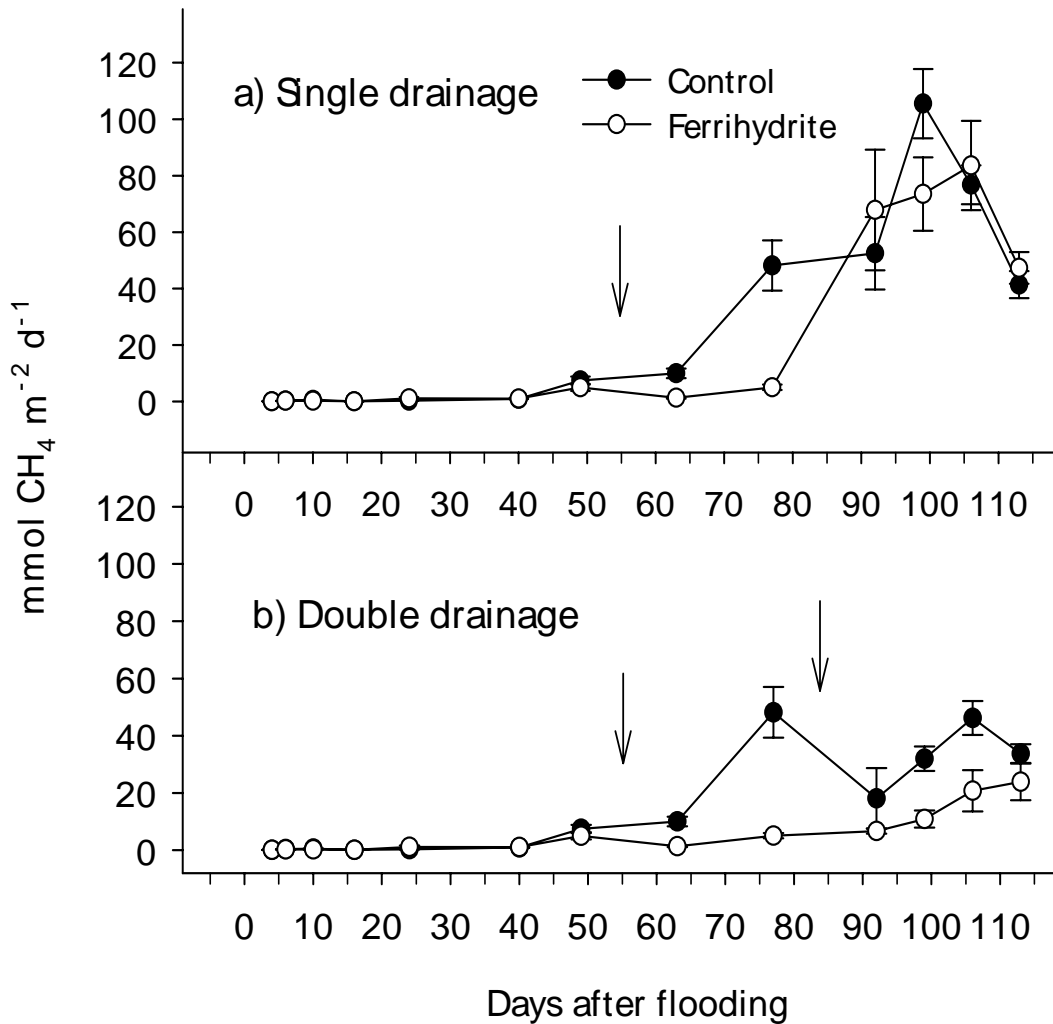


**Fig. 7.2** Fluxes of  $\text{N}_2\text{O}$  in the potted soil.

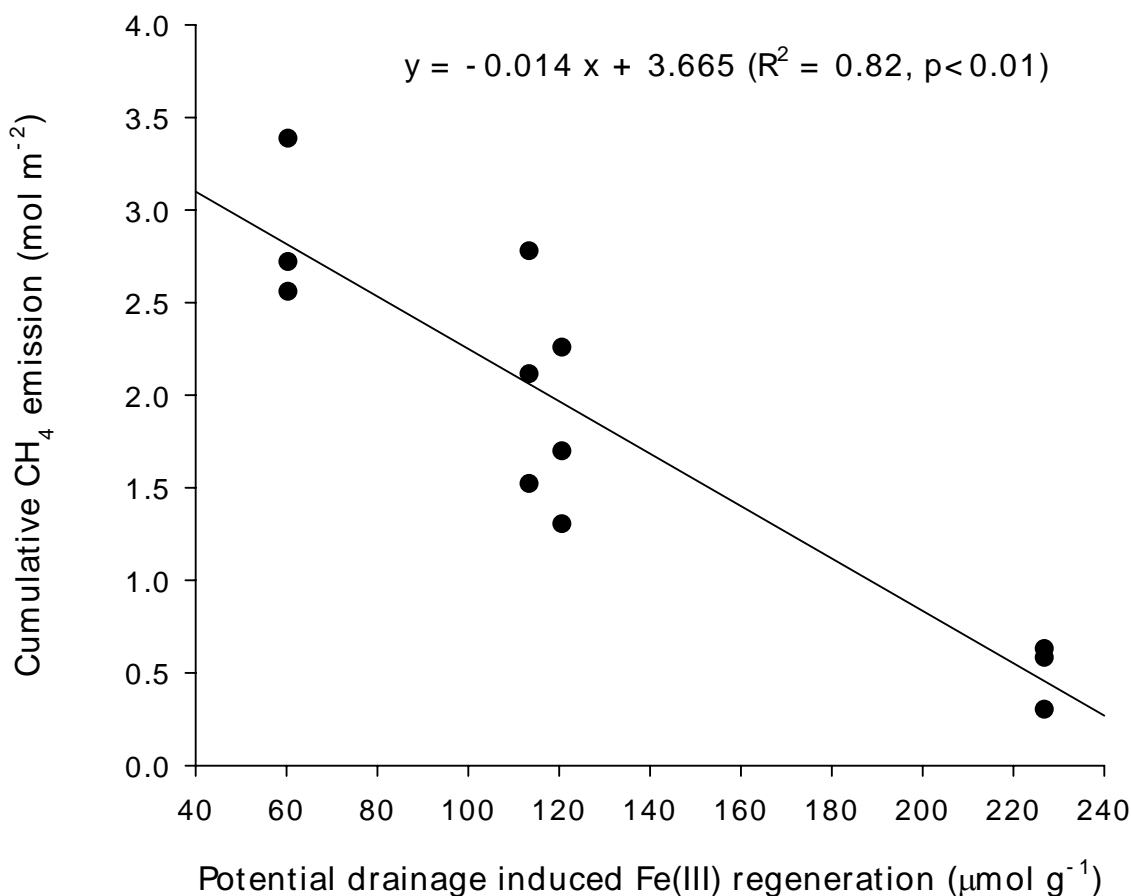
The initiation of  $\text{CH}_4$  emissions from the pots was relatively slow. Methane was emitted at low rates of less than  $1.5 \text{ mmol CH}_4 \text{ m}^{-2} \text{d}^{-1}$  in all the pots until day 40 after flooding (Fig. 7.3). Iron amendment had little effect on delaying and reducing  $\text{CH}_4$  emission before the midseason drainage on day 55.

Methane emission from the amended pots was significantly lowered by the single midseason drainage compared to the control pots through day 77 ( $p < 0.05$ ). Thereafter, similar high fluxes of more than  $40 \text{ mmol CH}_4 \text{ m}^{-2} \text{d}^{-1}$  were observed in both treatments on most dates. Ferrihydrite amendment generally lowered  $\text{CH}_4$  emission when the pot was drained twice until the end of measurements (Fig. 7.3). Seasonal  $\text{CH}_4$  emission was

reduced by 26% from 2.94 to 2.19 mol m<sup>-2</sup> by the single drainage (p=0.47) with ferrihydrite amendment and 69% from 1.81 to 0.56 mol m<sup>-2</sup> with the double drainage treatment (p < 0.01). A linear equation  $y = -0.014 x + 3.665$  ( $R^2 = 0.82$ ; p<0.01) was obtained (Fig. 7.4) between the potential drainage-induced Fe<sup>3+</sup> regeneration and the cumulative CH<sub>4</sub> emission during the 113-day measurement.



**Fig. 7.3** Methane emission from the potted soil affected by ferrihydrite amendment before and after drainage. The arrows show the dates when drainage was carried out.



**Fig. 7.4** Relationship between potential of drainage induced  $\text{Fe}^{3+}$  regeneration and cumulative  $\text{CH}_4$  emission (113 days) in the rice pots. The potential  $\text{Fe}^{3+}$  regeneration during each 2-day drainage was calculated as 100% regeneration of reducible iron in the soil, which was calculated according to the reducibility of indigenous soil iron and/or ferrihydrite from the slurry experiment (see Table 2.2 in Chapter 2).

### Rice Growth

The growth of rice seedlings was delayed by ferrihydrite amendment in the first four weeks after transplanting. No observable differences were found in rice growth between the control and amended pots in other periods. Rice yield and aboveground biomass (including rice seeds) in the amended pots were  $9.3 \text{ g} \pm 0.8$  and  $35.5 \text{ g} \pm 1.5$  per pot, not

significantly different from  $9.4 \text{ g} \pm 0.7$  ( $p = 0.92$ ) and  $34.6 \text{ g} \pm 0.3$  per pot ( $p = 0.52$ ) in the control pots, respectively.

## Discussion

In the potted soil,  $\text{NO}_3^-$  that accumulated under dry conditions contributed to  $\text{NO}_3^-$  percolation, but denitrification and  $\text{N}_2\text{O}$  emission were promoted in the early days after flooding. The effects of ferrihydrite amendment on increasing  $\text{NO}_3^-$  percolation and  $\text{N}_2\text{O}$  emission were consistent with those found in soil column and slurry experiments (Chapters 4 and 2). Nitrous oxide emission may be increased during short-term drainage (Ratering and Conrad, 1998). However,  $\text{NO}_3^-$  generated during the short-term drainage could only be transitionally accumulated due to rapid denitrification/utilization and hence, no high  $\text{N}_2\text{O}$  emission occurred soon upon reflooding.

While ferrihydrite amendment considerably reduced  $\text{CH}_4$  production in the same soil for 23 days under an anoxic slurry incubation (Chapter 2), it had little effect on  $\text{CH}_4$  emission from pot experiments for 40 days after flooding. Methane emission from the soil-rice system is the net result of  $\text{CH}_4$  production and oxidation, greatly mediated by the rice plant. Rice influence on production and transport of  $\text{CH}_4$  in the pots in the early period was expected to be weak as found by Aulakh et al (2000, 2001). The soil, without organic amendment, may not provide sufficient energy sources to support high  $\text{CH}_4$  production, but  $\text{CH}_4$  oxidation was expected to be active in this period (Krüger et al., 2002). A large part of  $\text{CH}_4$  produced in this period would be oxidized before it diffused or was transported into the atmosphere, leading to the low emission rates in all pots.

Thus, ferrihydrite amendment may reduce CH<sub>4</sub> production as found under anoxic incubation, but had only a small effect on reducing CH<sub>4</sub> emission from the pots in the early season.

However, ferrihydrite amendment significantly improved short-term drainage-induced CH<sub>4</sub> mitigation in the mid- and late-seasons when high CH<sub>4</sub> fluxes occurred. Drainage-induced CH<sub>4</sub> mitigation was mainly due to the toxic/inhibitory effects on methanogens of drainage-induced deeper O<sub>2</sub> diffusion, aerobic carbon oxidation and, generation and regeneration of AEAs (Sigren et al., 1997; Ratering and Conrad, 1998). Ferric iron is the dominant AEA capable of regeneration. The generation and regeneration of other AEAs such as NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> in the soil is likely to be less important. The onset of redox conditions to support methanogenesis upon reflooding was largely controlled by the amount of Fe<sup>3+</sup> generated during drainage. Ferrihydrite amendment at a relatively low rate of 3.5 g Fe per kg soil, compared to the rate of 10 or 20 g Fe per kg soil (15 or 30 g ferrihydrite per kg soil) applied by Jäkel and Schnell (2000), did not affect rice growth after the early weeks, but almost doubled the soil reducible iron content. This presumably increased Fe<sup>3+</sup> regeneration during drainage, resulting in more effective delay and reduction of CH<sub>4</sub> emission upon reflooding. This was also supported by the negative relation between the potential of drainage-induced Fe<sup>3+</sup> regeneration and the cumulative CH<sub>4</sub> mitigation.

When short-drainage is effective in CH<sub>4</sub> mitigation (Yagi et al., 1996; Ratering and Conrad, 1998), it is more desirable than long-term drainage because it also lowers the risk

of reducing rice yield and increasing N<sub>2</sub>O emission (Yagi et al., 1997; Henckel and Conrad, 1998; Cai et al., 1999). Nevertheless, factors affecting the mitigation extent need to be more carefully evaluated to ensure effective CH<sub>4</sub> mitigation by short-term drainage. In general, iron redox cycling in rice paddies is poorly quantified. More studies are needed for better evaluation of the role of Fe<sup>3+</sup> reduction and regeneration in CH<sub>4</sub> biogeochemistry and short-term drainage-based CH<sub>4</sub> mitigation in rice paddies.

### **Conclusions**

Microbial Fe<sup>3+</sup> reduction as a major alternative electron accepting process affected NO<sub>3</sub><sup>-</sup> dynamics and methanogenesis in the potted soil. An increase in reducible Fe<sup>3+</sup> content by ferrihydrite amendment temporally but significantly increased NO<sub>3</sub><sup>-</sup> percolation and N<sub>2</sub>O flux in the early days after flooding. Ferrihydrite amendment had little effect on CH<sub>4</sub> emission in the early growing season when CH<sub>4</sub> flux is low. Instead, ferrihydrite amendment effectively improved short-term drainage-induced CH<sub>4</sub> mitigation through increasing Fe<sup>3+</sup> regeneration in the mid- and late-seasons. Hence, the intensity of Fe<sup>3+</sup>-regeneration should be considered in short-term drainage-based options for effective CH<sub>4</sub> mitigation in rice paddies.

## **CHAPTER 8**

### **SUMMARY AND FUTURE RESEARCH RECOMMENDED**

#### **Summary**

Dissimilatory microbial  $\text{Fe}^{3+}$  reduction was a major intermediate alternative electron accepting process in anaerobic carbon decomposition. Controls on  $\text{NO}_3^-$  reduction and  $\text{CH}_4$  formation by iron-centered intermediate redox processes occurred to various extents in the soil slurry, soil column and rice pot experiments.

The roles of microbial  $\text{Fe}^{3+}$  reduction in denitrification and methanogenesis were first investigated in the homogenized anoxic soil slurries (Chapter 2). Ferric iron reduction was a major redox process in anaerobic carbon decomposition. More reducible iron and less favorable pH caused slower redox potential decrease, slower denitrification with more  $\text{N}_2\text{O}$  production, and later methanogenesis in the Texas soil compared to the Louisiana soil. An increase in  $\text{Fe}^{3+}$  reduction through  $\text{Fe}^{3+}$  amendment temporally stimulated  $\text{N}_2\text{O}$  production and considerably suppressed  $\text{CH}_4$  production in both soils, but had a small effect on the redox range within which both  $\text{N}_2\text{O}$  and  $\text{CH}_4$  are produced at low rates.

The roles of cycling of  $\text{Fe}^{3+}$ -centered AEAs in  $\text{N}_2\text{O}$  and  $\text{CH}_4$  production were also investigated in flooded soil columns (Chapter 3). Under the influence of  $\text{O}_2$  diffusion, iron-centered intermediate redox processes occurred around the aerobic/anaerobic interface and subsequently lowered methanogenic activity at upper depths. Nitrous oxide production was generally low in the soil column due to  $\text{NO}_3^-$  depletion. While under the



additional influence of downward diffusion of  $\text{NO}_3^-$ , iron-centered intermediate redox processes were vertically expanded and temporally extended, and caused relatively high  $\text{N}_2\text{O}$  production even at Eh levels around 0 mV partly due to intensive interactions of  $\text{Fe}^{2+}$  reoxidation with  $\text{NO}_3^-$  reduction, and, significantly suppressed methanogenesis to deeper depths for prolonged periods largely due to lowered substrate availability to methanogens.

Another flooded soil column experiment was carried out to investigate whether the competition for carbon sources of iron-centered intermediate redox processes in the flooded soil column would affect the fate of  $\text{NO}_3^-$  in the overlying water with and without percolation (Chapter 4). Moderate percolation caused the occurrence of  $\text{NO}_3^-$  percolation but promoted  $\text{NO}_3^-$  removal and lowered  $\text{N}_2\text{O}$  emission. Amendments of iron and manganese oxides decreased  $\text{NO}_3^-$  removal but promoted  $\text{N}_2\text{O}$  emission and  $\text{NO}_3^-$  percolation, primarily through intensifying iron-centered intermediate redox processes to lower carbon source availability, especially at the deeper depths, to heterotrophic denitrification. The pool of  $\text{Fe}^{3+}$ -centered AEAs needs to be considered in evaluation of  $\text{NO}_3^-$  removal and  $\text{N}_2\text{O}$  emission in seasonally and intermittently flooded wetlands.

Microbial activity involved in reduction of  $\text{Fe}^{3+}$ -centered intermediate AEAs was estimated using substrate induced respiration from a  $\text{Fe}^{3+}$ -reduction dominated intermediately reducing zone with comparison to other redox zones (Chapter 5). Acetate compared to glucose induced more  $\text{CO}_2$  production in the intermediate reducing zone, due to the dominance of obligate anaerobes and the weak role of fermenting activity,

which was active and responsible for comparable stimulation of  $\text{CH}_4$  production in highly reducing zones by acetate and glucose additions. Measuring both acetate and glucose induced respiration may be useful to account for the effect of redox conditions on microbial activity.

Ferric iron reducers are bioelectrochemically active microorganisms capable of using electrodes as electron donors and acceptors. Nitrate is a strong oxidant.

Bioelectrochemical reduction of  $\text{NO}_3^-$  in the overlying water was also investigated through a fuel cell process without direct contact with the flooded soil, the source of reductants (Chapter 6). Nitrate removal was faster under bioelectricity generation than under no electricity generation suggesting the applicability of a wide range of organic carbon sources for bioelectrochemical  $\text{NO}_3^-$  removal from drinking water. The current varied with microbial  $\text{NO}_3^-$  reduction and  $\text{N}_2\text{O}$  accumulation in the cathode chamber affected by the inoculation rate. When the enzymatic reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  was inhibited by acetylene, the current generated was higher than that coupled to  $\text{O}_2$  reduction.

The effect of  $\text{Fe}^{3+}$  reduction and regeneration in  $\text{NO}_3^-$  percolation and fluxes of  $\text{N}_2\text{O}$  and  $\text{CH}_4$  in a soil-rice system was investigated in the pot experiment (Chapter 7). Nitrate percolation and  $\text{N}_2\text{O}$  emission were stimulated by  $\text{Fe}^{3+}$  amendment in the early days after flooding. The effect of  $\text{Fe}^{3+}$  amendment on reducing  $\text{CH}_4$  emission was small in the early season when  $\text{CH}_4$  production was low, but was significant after 2-day drainage intervals in the mid- and late-seasons. The improved  $\text{CH}_4$  mitigation was mainly attributed to more

Fe<sup>3+</sup> regeneration during drainage in amended pots, delaying the onset of favorable soil conditions to support CH<sub>4</sub> production upon reflooding. No side effect of Fe<sup>3+</sup> amendment on rice yield was observed. This experiment shows that Fe<sup>3+</sup> regeneration plays an important role in short-term drainage-based CH<sub>4</sub> mitigation in rice soils.

### **Conclusions**

Reduction of Fe<sup>3+</sup>-centered intermediate AEAs was mainly mediated by obligate anaerobes relying on fermentation products.

Ferric iron reducers are important as bioelectrochemically active microorganisms, supporting bioelectricity generation from the flooded soil coupled to the reduction of oxidants O<sub>2</sub> and NO<sub>3</sub><sup>-</sup> in the overlying water.

Microbial Fe<sup>3+</sup> reduction was a major electron accepting process in flooded rice soils.

Ferric iron reduction temporally affected N<sub>2</sub>O production but its interaction with NO<sub>3</sub><sup>-</sup> reduction was obscured in homogenized soil slurries. In heterogeneous soil columns and rice pots, intensification of iron-centered intermediate redox processes changed the fate of NO<sub>3</sub><sup>-</sup> in the overlying water, decreasing heterotrophic denitrification and increasing NO<sub>3</sub><sup>-</sup> percolation and N<sub>2</sub>O emission.

Ferric iron reduction competitively suppressed methanogenic activity in homogenized soil slurries. Temporal and vertical variations of iron-centered intermediate redox processes subsequently controlled temporal and vertical variations of methanogenic activity in flooded soil columns. In a soil-rice system, Fe<sup>3+</sup> regeneration effectively reduced CH<sub>4</sub> emission after midseason drainage intervals.

### **Future Research Recommended**

The importance of cycling of  $\text{Fe}^{3+}$  in anaerobic carbon decomposition and  $\text{CH}_4$  emission in wetland environments, especially rice fields, has been acknowledged in recent studies. Nevertheless, reliable quantitative evaluation and predication of cycling of  $\text{Fe}^{3+}$  in wetland environments is yet to be established. Studies on cycling of  $\text{Fe}^{3+}$  at the soil-water interface and the rhizosphere in field conditions are needed to account for the role of cycling  $\text{Fe}^{3+}$  in anaerobic carbon mineralization and  $\text{CH}_4$  emission in rice fields.

The role of non-nitrate AEAs is poorly understood in denitrification in wetland environments. However, due to soil heterogeneity, AEAs other than  $\text{NO}_3^-$  do affect fate of  $\text{NO}_3^-$  in the flooded soil, especially under carbon limitation. Studies under more realistic conditions are needed to evaluate these effects on the fate of  $\text{NO}_3^-$  in seasonally and intermittently flooded wetlands, typically rice fields.

The possibility of using a wide range of organic carbon sources for bioelectrochemical removal of oxidized pollutants, including  $\text{NO}_3^-$  from drinking water, also deserves further investigation.

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## VITA

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