Rice plants, drainage and crop rotation influence the methanogenic community in rice field soil

Dissertation

Zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

Dem Fachbereich Biologie

der Philipps-Universität Marburg

vorgelegt von

Björn Breidenbach

aus Hanau

Marburg an der Lahn 2015

Die Untersuchungen zu folgender Arbeit wurden von Januar 2012 bis März 2015 unter der Leitung von Prof. Dr. Ralf Conrad am Max-Planck-Institut für terrestrische Mikrobiologie in Marburg/Lahn durchgeführt.

Die Natur hat jederzeit recht, und das gerade am gründlichsten, wo wir sie am wenigsten begreifen.

- Johann Wolfgang von Goethe

Vom Fachbereich Biologie der Philipps-Universität Marburg als Dissertation angenommen am:

Erstgutachter: Prof. Dr. Ralf Conrad

Zweitgutachter: Prof. Dr. Michael Bölker

Tag der Disputation: 11.05.2015

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Publikationen veröffentlicht bzw. zur Veröffentlichung vorgesehen:

Breidenbach B, Pump J, and Dumont MG. The influence of rice plants on the microbial community structure in flooded paddy soil at different growth stages (*in preparation*)

Breidenbach B and Conrad R (2015). Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Frontiers in Microbiology* 5:752. doi: 10.3389/fmicb.2014.00752

Breidenbach B, Blaser M, Klose M and Conrad R. Crop rotation of flooded rice with upland maize impacts resident and active methanogenic community (*in preparation*)

Contents

Summary 1

Zusammenfassung 4

1) Introduction 7

1.1 Atmospheric methane 7

1.2 Methanogenic Archaea 10

1.3 Biogeochemistry in rice fields 13

1.4 How plants influence microbes in the rhizosphere 16

1.5 Crop rotational systems 18

1.6 Aims of the study 21

1.7 References 22

2) *The influence of rice plants on the microbial community structure in flooded paddy soil at different growth stages* 38

2.1 Abstract 39

2.2 Introduction 40

2.3 Materials and methods 42

2.4 Results 46

2.5 Discussion 58

2.6 Supplemental material 63

2.7 Acknowledgements 64

2.8 References 65

3) Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage 72

3.1 Abstract 73

3.2. Introduction 74

3.3 Material and methods 76

3.4 Results 82

3.5 Discussion 98

3.6 Supplemental material 105

3.7 Acknowledgement 109

3.8 References 110

4) Crop rotation of flooded rice with upland maize impacts the resident and active methanogenic microbial community 121

4.1 Abstract 122

4.2 Introduction 123

4.3 Material and methods 126

4.4 Results 130

4.5 Discussion 146

4.6 Supplemental material 153

4.7 Acknowledgement 165

4.8 References 166

5) Discussion and concluding remarks 176

5.1 Microbial communities in the rice rhizosphere 178

5.2 Crop rotation 180

5.3 Increased rRNA levels - a potential stress response? 185

5.4 Outlook 187

5.5 References 190

Appendices 195

Wissenschaftliche Publikationen 195

Curriculum Vitae ..... **Error! Bookmark not defined.**

Abgrenzung der Eigenleistung 199

Erklärung 200

Danksagung 201

Summary

Rice fields significantly contribute to the global methane emission. The continuous flooding of rice fields results in anoxic conditions in the soil, creating an optimal habitat for anaerobic bacteria and methanogenic archaea. Furthermore, rice plants supply important nutrients for soil microbes by significantly contributing to the carbon pool by excreting carbon compounds through their root system. It is assumed that this supply of nutrients from the rice plants influences the microbial community structure and diversity, but this influence is poorly understood.

The first part of this thesis investigates the impact of the rice plant and its growth stages on the microbial community inhabiting flooded rice field soil. In a greenhouse experiment we showed that the presence of the rice plant leads to increased growth of both Archaea and Bacteria by detecting a doubling of the 16S rRNA gene copies. The overall microbial community composition was mainly similar in planted and unplanted soil. However, specific bacterial lineages were more abundant in the presence of the rice plant (e.g *Geobacter*, *Herbaspirillum* and *Opitutus*). In the planted soil major OTUs increased in relative abundance with plant growth stage, indicating that the rice growth stages and dynamics in root exudation influenced the microbial community. Together, these results suggest that the microbial community in the rice field soil is highly adapted to the presence of rice plants, possibly because of the plant-supplied carbon compounds in the soil.

The traditional method for rice cultivation is the flooding of the field. However, with the anticipated increase in the human population the demand on resources such as water will increase. Therefore, rice farmers will probably face periods of restricted water availability. A method decreasing the water demand of rice cultivation is the rotation with plants cultivated under upland conditions such as maize, which require less water. Therefore, the second part of this thesis deals with the influence of the rice plant growth stages, field conditions and maize cultivation on the microbial community in rice field soil. During the plant growth stages we detected only minor changes in abundance, composition and activity of both archaeal and bacterial communities. In contrast, changes in field management such as drainage and the cultivation of maize resulted in comparatively stronger changes in the bacterial community. Bacterial lineages that increased in relative abundance under non-

flooded conditions were either aerobes such as *Spartobacteria* and *Sphingobacteria* or were characterized by their ability to grow under low substrate conditions such as *Bacteroidetes* and *Acidobacteria.* Besides archaeal lineages commonly found in rice fields (*Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriaceae* and *Methanocellaceae*) we found notably high numbers of GOM Arc I species within the order of *Methanosarcinales*, which may be anaerobic methane oxidizers. The archaeal community remained mainly unchanged throughout the monitored season. Interestingly, we observed increased ribosomal RNA levels per cell under the drained conditions. As these conditions were unfavorable for anaerobic bacteria and methanogenic archaea we interpreted this behavior as preparedness for becoming active when conditions improve.

In the third part of the thesis we followed the introduction of maize cultivation and concomitant non-flooded conditions on fields that had previously been managed as flooded rice fields. The crop rotation was monitored for two additional years. Thereby we found only minor differences in the bacterial community abundance and activity in the rotational fields in comparison to flooded rice fields. *Acidobacteria* and *Anaeromyxobacter* spp. were enriched in the rotational fields while members of anaerobic *Chloroflexi* and sulfite reducing members of *Deltaproteobacteria* were found in higher abundance in the rice fields. In contrast, we showed that rotation of flooded rice and upland maize lead to dramatic changes in the archaeal community, indicated by a decrease of anaerobic methanogenic lineages and an increase of aerobic *Thaumarchaeota*. This was especially apparent in the strong enrichment of *Thaumarchaeota* of the Soil Crenarchaeotic Group, mainly *Candidatus* Nitrososphaera, indicating the increasing importance of ammonia oxidation during drainage. Combining qPCR and pyrosequencing data again revealed increased ribosomal numbers per cell for methanogenic species during crop rotation. This stress response, however, did not allow the methanogenic community to recover in the rotational fields during the season of re-flooding and rice cultivation.

Summary

This thesis provides evidence that the rice plants influence the microbial community in the soil (first part), and that alterations in field management such as drainage or maize cultivation under upland conditions have minor immediate effects on the overall microbial community (second part) but more strongly pronounced long term effects mainly on the archaeal community (third part).

Zusammenfassung

Reisfelder bieten ein optimales Habitat für anaerobe Bakterien und methanogene Archaeen, die wesentlich zur globalen Methanemission beitragen. Reisfelder zeichnen sich durch Flutung während des Reisanbaus aus, was die Bildung von anoxischen Nischen begünstigt. In diesen Nischen findet der anaerobe Abbau von organischem Material bis hin zur Bildung von Methan durch methanogene Archaeen statt. Ein wesentlicher Teil des organischen Materials in Reisfeldern stammt von der Reispflanze selbst, welche Kohlenstoffverbindungen über ihr Wurzelsystem ausscheidet.

Im ersten Teil dieser Arbeit wurde der Einfluss der Reispflanze auf die mikrobielle Gemeinschaft im Boden untersucht. Die bakterielle Gemeinschaft unterschied sich dabei nur geringfügig in ihrer Zusammensetzung zwischen bepflanztem und unbepflanztem Reisfeldboden. Dabei zeigten unter anderem *Geobacter*, *Herbaspirillum* and *Opitutus* eine höhere Abundanz im bepflanzten Boden. Während sich die Anzahl der Bakterien und der Archaeen im bepflanzten Boden verdoppelte, zeigte die Zusammensetzung der archaeellen Gemeinschaft wenig Veränderung. Über den Zeitraum des Pflanzenwachstums zeigten wenige bakterielle Gruppen eine Veränderung in ihrer relativen Abundanz. Dies weist auf einen möglichen Einfluss der Pflanze hin, da sich deren Wurzelexudate in Qualität und Quantität während der Wachstumsphasen unterscheidet. Zusammengefasst zeigten die Ergebnisse jedoch, dass die mikrobielle Gemeinschaft im Reisfeldboden stark an die Reispflanze und deren Wurzelexudation adaptiert ist.

Reis wird traditionell unter gefluteten Feldbedingungen angebaut. Aufgrund der stetig steigenden Weltbevölkerung ist ein Anstieg der Nachfrage für Ressourcen wie Wasser anzunehmen. Dies könnte eine eingeschränkte Verfügbarkeit an Wasser für den Reisanbau zur Folge haben. Alternative Anbaustrategien, die eine Reduzierung des Wasserverbrauchs im Vergleich zum konventionellen Reisanbau ermöglichen, rücken somit immer mehr in den Fokus. Der Fruchtfolgewechsel mit einer Pflanze wie Mais, die unter nicht gefluteten Feldbedingungen wächst, ist eine dieser Optionen. Der zweite Teil dieser Arbeit unterteilt sich daher in zwei Schwerpunkte: Unter Feldbedingungen wurde der Einfluss (I) der Wachstumsstadien der Reispflanze sowie (II) der von Feldbearbeitungsmaßnahmen wie der Dränage und dem Anbau von Mais unter nicht gefluteten Bedingungen auf die mikrobielle

Gemeinschaft im Reisfeldboden untersucht. Dabei wurde gezeigt, dass sich die Wachstumsstadien der Reispflanze nur begrenzt auf die Zusammensetzung und Aktivität der Mikroben auswirkten. Im Gegensatz dazu führten Dränage und der Anbau von Mais zu einer Abnahme der Abundanz der Mikroben. Desweiteren zeigten verschiedene bakterielle Gruppen eine Reaktion auf die Feldbearbeitungsmaßnahmen indiziert durch eine erhöhte relative Abundanz in den nicht gefluteten Feldern. Diese unterteilten sich in zwei Gruppen: (I) aerobe Organismen wie *Spartobacteria* und *Sphingobacteria* und (II) Bakterien, die unter substratlimitierten Bedingungen wachsen, wie *Bacteroidetes* und *Acidobacteria*. Im Gegensatz dazu blieb die archaeele Gemeinschaft weitestgehend unbeeinflusst. Generell konnten auch in dieser Arbeit reisfeldtypische Methanogene wie *Methanosarcinaceae, Methanosaetaceae*, *Methanobacteriaceae* und *Methanocellaceae* gefunden werden. Interessanterweise wurden innerhalb der Ordnung der *Methanosarcinales* eine große Anzahl an GOM Arc I Spezies gefunden, die potentiell zur anaeroben Methanoxidation fähig sind. Desweiteren, wurde während der nicht gefluteten Bedingungen beobachtet, dass der Ribosomengehalt pro Zelle auf einem hohen Niveau gehalten wurde. Dies wurde als Stressantwort aller anaeroben Archaeen und Bakterien auf die ungünstigen aeroben Bedingungen interpretiert.

Im dritten Teil dieser Arbeit wurden der Verlauf des Fruchtfolgewechsels und dessen Einfluss auf die mikrobielle Gemeinschaft im Boden über einen Zeitraum von zwei weiteren Jahren verfolgt. Nach Einführung der Maiskultivierung in das Reisökosytem erfolgte ein jährlicher Fruchtfolgewechsel mit Reis (geflutet) in der Regenzeit und Mais (nicht geflutet) in der Trockenzeit. Alternativ wurde in beiden Jahreszeiten Reis unter gefluteten Bedingungen angebaut. Die bakterielle Gemeinschaft zeigte eine geringe Reaktion auf den Fruchtfolgewechsel. *Acidobacteria* und *Anaeromyxobacter* sp. waren in den Fruchtfolgewechselfeldern angereichert, während anaerobe *Chloroflexi* und sulfatreduzierende *Deltaproteobacteria* in höherer Abundanz in den Reisfelder gefunden wurden. Eine stärkere Veränderung erfolgte in der archaeellen Gemeinschaft. Ausgehend von einer von methanogenen Archaeen dominierten Gemeinschaft in den gefluteten Reisfeldern entwickelte sich in den nicht gefluteten Maisfeldern eine überwiegend aus aeroben Thaumarchaeoten bestehende Gemeinschaft. Innerhalb der Thaumarchaeoten wurde die Gruppe Soil Crenarchaeotic Group angereichert, welche hauptsächlich von

Ammoniumoxidierern (*Candidatus* Nitrososphaera) repräsentiert wurde. Dies deutet darauf hin, dass der Oxidation von Ammonium möglicherweise eine höhere Bedeutung in nicht gefluteten Boden zukommt. Desweiteren, zeigten auch hier die methanogenen Euryarchaeoten unter nicht gefluteten Feldbedingungen eine Stressreaktion in Form einer erhöhten Ribosomenzahl pro Zelle. Das erneute Fluten in der Regenzeit ermöglichte den Euryarchaeoten allerdings nicht, sich wieder zu erholen und ihre ursprüngliche Abundanz zu erreichen.

Die Ergebnisse dieser Arbeit lassen annehmen, dass die Reispflanze Einfluss auf die Mikroben im Reisfeldboden nimmt (erster Teil). Desweiteren führten Veränderungen in den Feldbearbeitungsmaßnahmen, wie Dränage und Fruchtfolgewechsel, zu einer schwachen kurzfristigen Reaktion der gesamten mikrobiellen Gemeinschaft (zweiter Teil) und einer stärkeren langfristigen Reaktion der methanogenen Archaeen (dritter Teil).

Chapter 1

Introduction

1.1 Atmospheric methane

Methane (CH_4) is a simple colorless, odorless and volatile hydrocarbon gas. This flammable gas was first discovered by Allesandro Volta in the year 1776. At the Lake Maggiore in Italy he observed this very flammable gas rising up when the shallow sediment was disturbed. Today methane is recognized as the second most important anthropogenic greenhouse gas after carbon dioxide (CO_2), having a 25 times larger global warming potential than CO_2 (Forster *et al*., 2007). The lifetime of methane in the atmosphere is about eight years and the global budget of atmospheric methane is in the order of 500-600 Tg per year (Denman *et al*., 2007; Forster *et al*., 2007; Thauer, 2011). Methane concentrations in the atmosphere varied between 350-800 ppbV in the past 800,000 years and increased dramatically in the last 250 years up to the present concentration ~1800 ppbV, mainly caused by increasing anthropogenic activity (Spahni *et al*., 2005; Loulergue *et al*., 2008; Forster *et al*., 2007; Hartmann *et al*., 2013; Figure 1.1A).

Once methane is emitted into the atmosphere most of it is removed through chemical oxidation with hydroxyl radicals in the troposphere, while a minor part is lost to the stratosphere. An additional sink for methane is biological oxidation in e.g. upland soils. Methane emissions highly vary from year to year and great uncertainties regarding the global budget are recognized.

The largest sources of atmospheric methane are natural wetlands, which emit together with termites, oceans and hydrates between 145-260 Tg CH_4 per year (Forster *et al*., 2007; Figure 1.1C). Besides these natural sources the majority of the emitted methane is of anthropogenic origin. Sources like landfills, biomass burning, rice agriculture, ruminant animals and energy generation account for up to 70% of the total global budget (Lowe, 2006; Figure 1.1C).

Biogenic methane is solely formed by the process of anaerobic methanogenesis which is conducted by a particular guild of microorganisms, the methanogenic Archaea.

Recently, non-biogenic methane emissions from plant leaves were observed (Keppler *et al*., 2006).

Here, the mechanism of methane formation is believed to proceed by photochemical cleavage of methyl groups of pectin found in leaf tissues by UV radiation (McLeod *et al*., 2008). Nevertheless, this methane source has not been integrated in the majority of the global methane balances due to uncertainties in the estimated amounts.

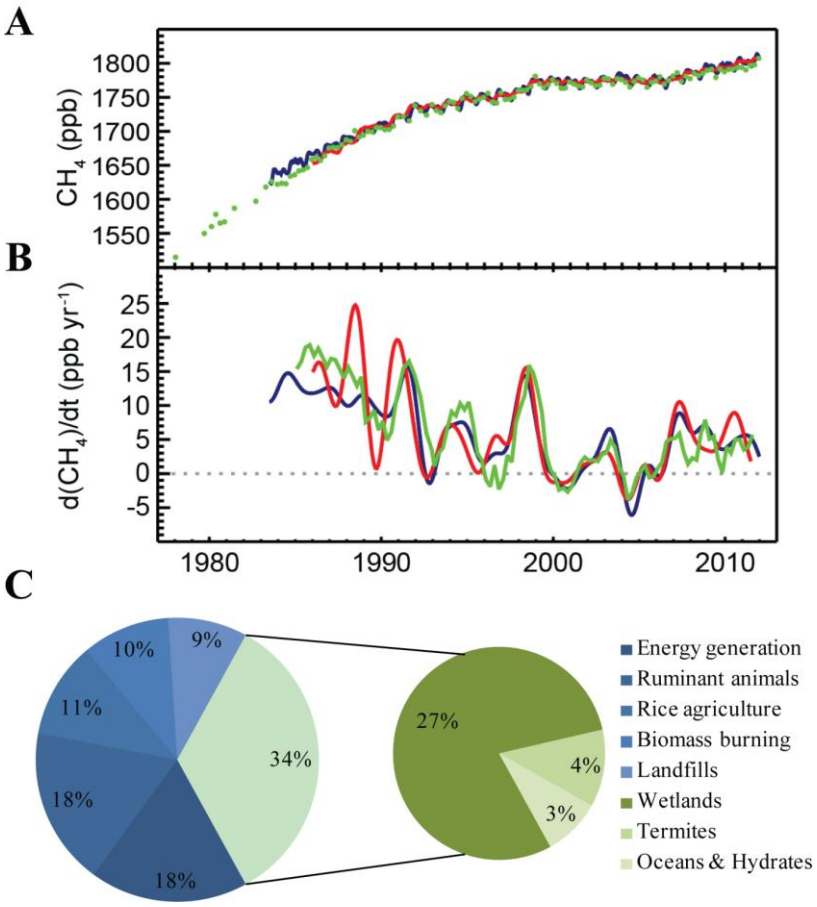


Figure 1.1 Atmospheric methane concentration and its sources. (A) Atmospheric methane concentration determined weekly (blue), monthly (red) and every quarter year (green). (B) Growth rate of atmospheric methane in the global average using the same color code as in (A) (figure adapted from Hartmann *et al*., 2013). (C) Anthropogenic (blue) and natural (green) sources of atmospheric methane (data from Lowe, 2006).

A decreasing growth rate of methane in the atmosphere was reported from the early 1980s until 1998 followed by a stabilization from 1999 to 2006 and an increase from 2007 to 2011(Rigby *et al*., 2008; Dlugokencky *et al* 2009; Figure 1.1B). Potential missing sources (e.g. plant leaves) beside changes in agricultural practices or the decline in fossil-fuel can explain the variability observed (Kai *et al*., 2011; Aydin *et al*., 2011). While the rise of methane in the atmosphere seemed to be ceased since the late 1980s a recent increase was observed (Dlugokencky *et al*., 1998; Rigby *et al*., 2008). Suggested drivers of the increase in atmospheric methane were atypically elevated temperatures in the Arctic in 2007 and increased precipitation in the tropics during 2007 and 2008 (Dlugokencky *et al*., 2009; Bousquet *et al*., 2006).

1.2 Methanogenic Archaea

Methanogenic archaea are characterized by their ability to gain energy by producing CH_4. All known methanogens belong to the phylum *Euryarchaeota* and are confined to use methanogenesis as sole process for their energy metabolism (Whitman *et al*., 2006). The methanogens are phylogenetically divers. Until now seven orders of methanogens are described, namely *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales* and the newly described *Methanomassiliicoccales* (Narihiro and Sekiguchi, 2011; Borrel *et al*., 2014).

Methanogens are found in a large variety of environments characterized by limited electron acceptors like oxygen (O_2), nitrate (NO_3^-), manganese (Mn^{4+}), iron(III) (Fe^{3+}) and sulfate (SO_4^{2-}) including flooded soils, freshwater and marine sediments, termites, human and animal gastrointestinal tracts, anaerobic digesters and landfills, geothermal systems and heartwood of trees (Liu and Whitman, 2008). Methanogens, independent of their diversity and large number of habitats, are limited in the substrates they are able to utilize. Three major groups are described as possible substrates: CO_2, acetate and methyl-group containing compounds (Table 1.1).

Table 1.1 Important methanogenic reactions and obtained free energy.

Reaction	$\Delta G°$ (kJ mol^{-1})	Organism
CO_2		
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-131 kJ	Most methanogens
Acetate		
$CH_3COOH \rightarrow CH_4 + CO_2$	-33 kJ	*Methanosarcina* and *Methanosaeta*
Methylated C1 compounds		
$4\ CH_3COH \rightarrow 3\ CH_4 + CO_2 + H_2O$	-105 kJ	Methylotrophic methanogens

Modified from Hedderich and Whitman, 2006; Liu and Whitman, 2008; Zinder 1993.

In rice field soils CH_4 is produced by two major physiological guilds of methanogens, the hydrogenotrophic and acetotrophic methanogens. The hydrogenotrophic methanogens reduce CO_2 with H_2 to CH_4 (Table 1.1), whereby CO_2 is reduced successively

to CH_4 through formyl, methylene and methyl levels. Acetotrophic methanogens convert acetate to CH_4 and CO_2 (Table 1.1). Here, acetate is split oxidizing the carboxyl-group to CO_2 and reducing the methyl-group to CH_4. Most methanogens are hydrogenotrophic using H_2 as electron donor, while some also can use formate as electron donor. However, further more exotic electron donors are found to be used by hydrogenotrophic methanogens such as secondary alcohols (e.g. 2-propanol, 2-butanol) and ethanol. Only a limited number of the known methanogens is able to use acetotrophic methanogens for their energy metabolism namely *Methanosaeta* and *Methanosarcina*. Since acetate is a major intermediate in the anaerobic food chain in many environments two-thirds of the produced CH_4 is acetotrophically generated (Liu and Whitman, 2008).

Despite the different substrates, the complex pathway of methanogenesis encloses key enzymes and coenzymes shared by all methanogens. These are tetrahydromethanopterin, F_{420}-hydrogenase and coenzyme M. Coenzym M is fundamental for the last step of methanogenesis. In this last step the methyl-group bound to coenzyme M is reduced by the hydrogen provided by a reduced coenzyme B. This reaction is catalyzed by the methyl-coenzyme M reductase (Mcr), which is the characteristic enzyme unique to all known methanogens (Thauer, 1998). The methyl-coenzyme M reductase is relatively well conserved and homologous in all methanogens and therefore the α subunit of the encoding gene (*mcrA*) is used as functional genetic marker to identify methanogens in the environment (Friedrich, 2005; Luton *et al*., 2002).

In addition the archaeal 16S rRNA gene as marker gene for Archaea has been extensively used along with *mcrA* to study methanogenic archaea in rice field soil demonstrating a worldwide distribution (China, Italy, Japan and Philippines) of acetoclastic and hydrogenotrophic methanogens including members of *Methanosarcinaceae*, *Methanobacteriales*, *Methanomicrobiales* and *Methanocellales* (former RC-I) (Grosskopf *et al*., 1998; Ramakrishnan *et al*., 2001; Wu *et al*., 2006). In rice fields, *Methanocellales* are of great importance since they are the key CH_4 producers in the rice rhizosphere (Lu and Conrad, 2005).

Methanogens show a specific style of stress resistance which stands in line with the suggestion that chronic energy stress is the primary selective pressure pushing the evolution

of Archaea (Valentine, 2007). Methanogens are known as strict anaerobes and harbor numerous enzymes with oxygen-sensitive redox centers (Jarrell, 1985). Consequently, methanogenesis is completely suppressed by exposure to oxygen as demonstrated in pure cultures and soil samples (Fetzer *et al*., 1993; Fetzer and Conrad, 1993; Yuan *et al*., 2009). Despite these facts, several species, mainly *Methanosarcina* spp. and *Methanocella* spp., were frequently found in aerated soils such as pasture and barley soils and even in desert biological soils crusts (Nicol *et al*., 2003; Poplawski *et al*., 2007; Angel *et al*., 2012; Conrad *et al*., 2012; Aschenbach *et al*., 2013). Additionally, methanogens have been shown to survive oxygen stress in pure culture and soil (Fetzer *et al*., 1993; Ueki *et al*., 1997; Liu *et al*., 2008; Ma and Lu, 2011). As several methanogens possess a relatively large number of genes coding for oxygen-detoxifying enzymes (Erkel *et al*., 2006) these may function as defense mechanism during oxygen stress, possibly allowing them to survive oxygen exposition in dry soils (Angel *et al*., 2011, 2012).

1.3 Biogeochemistry in rice fields

Rice paddies represent a unique wetland type connected to the monoculture of rice plants. Wetlands are defined as ecosystems in which water saturation is the dominant factor determining soil development and composition of floral and faunal communities inhabiting the soil and its surface (Cowardin *et al*., 1979). In general, wetland rice agriculture is distinguished by the water profile of the fields. The three major types are (I) irrigated rice which is artificially flooded during the season and drained in the winter, (II) rain-fed rice which is only flooded after heavy rains and (III) permanently flooded deep water rice (Neue and Roger, 1993).

Methanogens are found in high abundance in the rice field ecosystem which's biogeochemistry makes it a suitable habitat for these anaerobic archaea. In rice paddies the biogeochemical cycle is controlled by a) the input of organic carbon, b) the redox conditions rendered by the availability of oxygen as well as alternative electron acceptors (e.g. Fe^{3+}, NO_3^-, Mn^{4+} and SO_4^{2-}) and c) an unique microbial community (Conrad and Frenzel, 2002). The organic carbon pool in rice paddies is composed of soil organic matter and the organic carbon originating from decayed plant material or released by the plant through root exudation (Hartmann *et al*., 2009). The impact of plant derived carbon on the microbes in the rice field soil will be explained in chapter 1.4.

Oxygen availability is one of the important factors characterizing flooded rice paddies. Upon flooding oxygen is rapidly consumed in the soil and cannot be replenished since the water layer limits the gas diffusion. Hence oxygen only penetrates the upper few millimeters of the soil (Frenzel *et al*., 1992). On the other hand the intercellular aerenchyma system of the rice plant allows an oxygen transport throughout the plant root to deeper anoxic soil compartments (Armstrong, 1979; Frenzel *et al*., 1992; Große and Bauch, 1991). The limitation in oxygen shapes flooded paddy rice fields into three distinct compartments (major habitats for microorganisms) namely the anoxic bulk soil, the oxic surface soil, and the partially oxic rhizosphere (Figure 1.2).

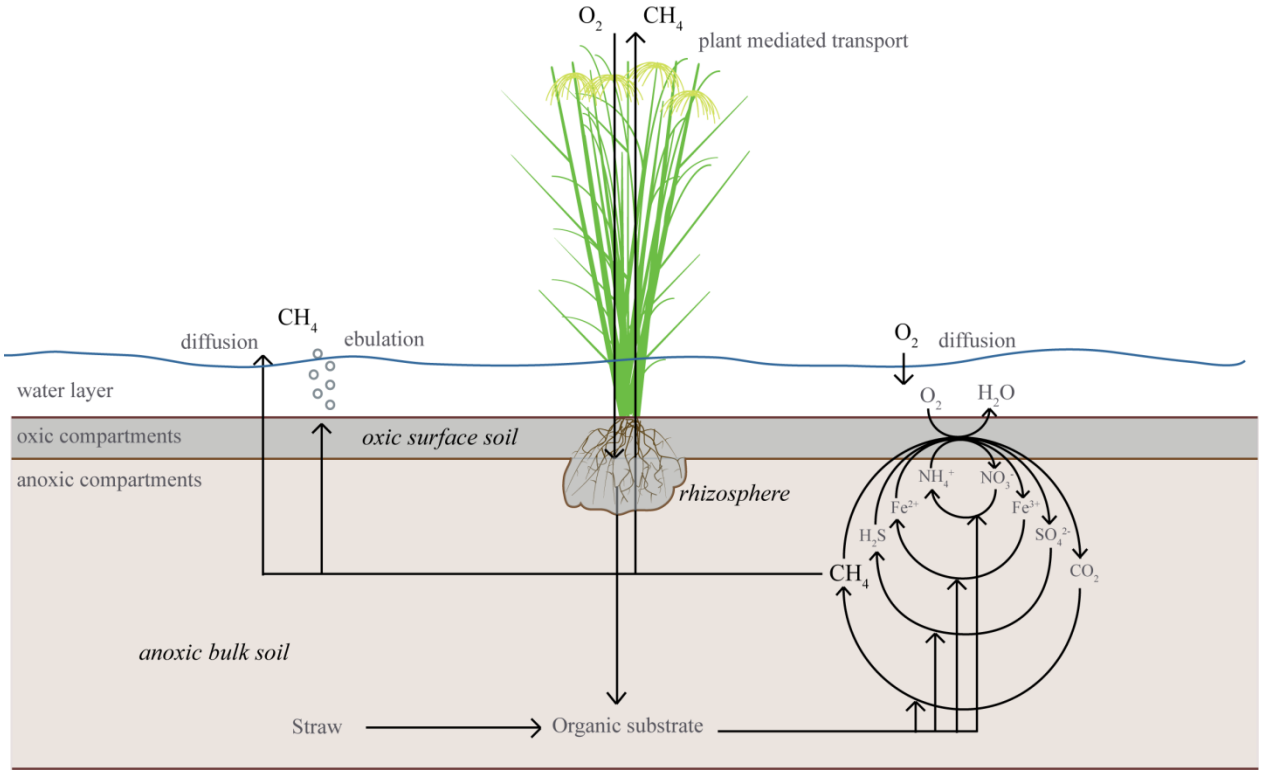


Figure 1.2 Microbial habitats in rice field soil. The three major microbial habitats namely the oxic surface soil, the rhizosphere and the anoxic bulk soil are presented in addition to a simplified presentation of the redox cycling and methane emission pathway. Modified from: Conrad, 2007; Conrad and Frenzel, 2002.

In upland soils the primary electron acceptor is oxygen. Organic carbon is completely oxidized to carbon dioxide. Under anoxic conditions the degradation of organic matter is more complex (Chin *et al*., 1998; Liesack *et al*., 2000; Figure 1.2): A cascade of alternative electron acceptors following the redox potential can be used instead of oxygen: NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-} (Patrick and Reddy, 1978; Ponnamperuma, 1972). After flooding, when oxygen and nitrate are rapidly depleted, Fe^{3+} reduction is the dominating reaction (Conrad and Frenzel, 2002; Figure 1.2). In paddy fields Fe^{3+} is the most prominent electron acceptor (Yao *et al*., 1999). These electron acceptors can be regenerated at oxic-anoxic interfaces predominantly at the surface soil and at the rhizosphere, where a redox cycling of nitrogen (N), iron (Fe) and sulfur (S) takes place (Conrad and Frenzel, 2002; Figure 1.2). Only if these electron acceptors are depleted methanogenesis as the last step in anaerobic organic matter degradation is initiated.

While CO_2 is the predominant end product of organic matter degradation in upland soils, various reduced intermediates can be found during the anaerobic degradation of organic matter: First polysaccharides are hydrolyzed by enzymes excreted by fermenting bacteria, which then convert the released monomeric sugars to alcohols, fatty acids and hydrogen (H_2) (Chin *et al*., 1998; Liesack *et al*., 2000). Alcohols and fatty acids can be converted by syntrophic bacteria to acetate, formate (H_2-CO_2) and CO_2. Homoacetogenic bacteria alternatively convert monomers like sugars directly to acetate (Liu and Whitman, 2008) or reduce CO_2 and H_2 to acetate. In the final degradation step acetate or H_2/CO_2 function as substrates for methanogens metabolizing it to CH_4 and in the case of acetate to CH_4 plus CO_2 (Liesack *et al*., 2000).

The generated CH_4 then serves as substrate for methanotrophic bacteria. These bacteria can be found in habitats where methane and oxygen gradients overlap, mainly in the rhizosphere and the soil surface (Bosse and Frenzel, 1997; Eller and Frenzel, 2001; Gilbert and Frenzel, 1995). Besides the aerobic also the anaerobic oxidation of CH_4 was observed in rice field soils. Several styles of anaerobic CH_4 oxidation have been discovered: The first discovery of anaerobic CH_4 oxidation was found to be coupled to sulfate reduction conducted by a syntrophic association between archaeal clades ANME-1, ANME-2 or ANME-3 and sulfate-reducing bacteria (Boetius *et al*., 2000; Cadwell *et al*., 2008; Harrison *et al*, 2009). Further the presence of denitrifying anaerobic methane-oxidizing (damo) bacteria was discovered (e.g.: Raghoebarsing *et al*., 2006; Ettwig *et al*., 2008). Recently, damo bacteria were identified as '*Candidatus Methylomirabilis oxyfera*' affiliated to the 'NC10' phylum (Ettwig *et al*., 2010). These organisms are able to couple anaerobic CH_4 oxidation to nitrite reduction with intercellular oxygen production (Ettwig *et al*., 2010; Zhu *et al*., 2010). Latest studies indicated ANME-2d to oxidize methane through reverse methanogenesis by using nitrate as terminal electron acceptor (Haroon *et al*., 2013). Thereby produced nitrite is reduced to dinitrogen gas by an anaerobic ammonium-oxidizing bacterium as a syntrophic partner (Haroon *et al*., 2013). The nitrite-dependent anaerobic CH_4 oxidation has been identified to occur in wetlands and rice fields (Hu *et al*., 2014; Zhu *et al*., 2014; Zhou *et al*., 2014).

1.4 How plants influence microbes in the rhizosphere

In Figure 1.2 it was shown that rice plants allow oxygen to diffuse into the soil surrounding the rice roots, called rhizosphere. The rhizosphere was first defined by Hiltner (1904) and comprises the soil surrounding living roots which is influenced by root activity. In these soil regions the plants have to compete with other plants and soil-born organisms (e.g. bacteria, fungi, insects) for space, water, minerals and macronurtrients such as carbon or nitrogen (Ryan and Delhaize, 2001; Bais *et al*., 2004). The rhizosphere is a highly populated spot in the soil characterized by various interspecies interactions. It was reported that plants have the ability to mediate both positive and negative interactions in the rhizosphere (Bais *et al*., 2006; Philippot *et al*., 2013). Positive interactions include symbiotic interactions with beneficial microbes or recruitment of plant promoting bacteria whereas negative interactions are characterized by associations with pathogenic microbes, parasitic plants and invertebrate herbivores (Bais *et al*., 2006; Haichar *et al*., 2014; Philippot *et al*., 2013). Specific plant-microbe interactions were reported for example relating to positive effects on the plant as on the plants immune system (Jones and Dangl, 2006) or for soil bacteria utilizing plant-born carbon (Dennis *et al*., 2010; Bais *et al*., 2006).

Plants release a large variety of compounds into the soil via their roots. During this process, the so called rhizodeposition, carbon and nitrogen compounds are released. The plant derived carbon release into the soil leads to changes in chemical, physical and biological characteristics in the rhizosphere soil which therefore can be differentiated from bulk soil (Barber and Martin, 1976). The compounds secreted by a plant into the rhizosphere are called root exudates. They are divided into low- and high-molecular weight compounds. Whereas the class of low-molecular weight compounds includes amino acids, organic acids and sugars, the latter one consists of polysaccharides and proteins (Badri and Vivanco, 2009). Thereby some plants are able to secrete significant amounts (up to 60%) of their photosynthetically fixed carbon into the rhizosphere (Derrien *et al*., 2004; Lynch and Whipps, 1990). The amount of root exudation may vary since the quality and quantity is a function of plant species, plant age as well as of external biotic and abiotic factors (Jones *et al*., 2004). Especially for rice plants it was shown that root exudation varied in composition and rate during the growth of the plants (Aulakh *et al*., 2001).

Root exudates represent the main part of the carbon released by plants into the soil (Hutsch *et al*., 2000; Nguyen, 2003). Thereby soluble low-molecular weight compounds diffuse passively from the root into the soil (Bertin *et al*, 2003; Bais *et al*., 2006). This diffusion is driven by a concentration gradient between the root and the soil. Diffusing compounds such as amino acids, organic acids, sugars or phenolics are present inside the root in significantly higher concentrations than in the surrounding soil as a result of the continuous replenishment inside the roots along with removal by the soil microbes (Jones *et al*., 2009).

However, root exudation is not the only way in which plants influence the soil and possibly the inhabiting microbes. Further important mechanisms are the mucilage and border cells. Mucilage is a gelatinous layer surrounding root tips mainly composed of polysaccharides, proteins and phospholipids (Jones *et al*., 2009; Read *et al*., 2003). Its main function is besides protecting roots of toxic metals, to enhance the stability of soil aggregates. This in consequence promotes root growth and soil aeration (Jones *et al*., 2009). Border cells are metabolically active root cells, programmed to be released from the root into the surrounding soil (Hawes *et al*., 2000; Stubbs *et al*., 2004).

Up to 90% of the excreted carbon were shown to be metabolized by the root-associated microorganisms (Lynch and Whip; 1990). Especially in rice field soil close interactions between plants and microbes were reported. A dominant part of the CH_4 emissions (~60%) originate from root exudates or dead roots (Watanabe *et al*., 1999). Pulse labeling experiments indentified methanogenic archaea incorporating plant derived carbon (Lu and Conrad, 2005) and showed correlations between photosynthesis driven CH_4 emissions and abundance of methanogens on the root (Pump *et al.*, 2014). Likewise bacterial lineages metabolizing plant derived carbon were identified inhabiting the rice roots and the surrounding rhizsophere soil (Hernández *et al*., 2015). Recently, the rice root-associated microbiome was investigated describing distinct communities in the endosphere (root interior), the rhizoplane (root surface) and the rhizsophere (Edwards *et al*., 2015).

1.5 Crop rotational systems

Rice (*Oryza sativa* L.) is a very important staple food feeding more than three billion people worldwide (Maclean *et al*., 2002). With the anticipated increase in the world's population the need for optimized rice cultivation will increase (Van Nguyen and Ferrero, 2006). Accordingly, the global paddy rice production area increased from circa 115 in 1961 to approximately 164 million hectare in 2013 underlining increasing demands (FAOSTAT, 2012).

The production of rice is intense in water usage since rice is generally cultivated under flooded field conditions. However, even in comparison with other irrigated crops rice requires enormous amounts of water (3,000 – 5,000 l/kg rice) as the water demand is tripled per hectare growing rice in comparison to other irrigated crops (Bouman *et al*., 2002, Tuong *et al*., 2005). Therefore up to one-third of the World's freshwater resources are used for rice cultivation (Bouman *et al*., 2007). The anticipated increase in world population will amplify the need for staple food like rice (Van Nguyen and Ferrero, 2006). To meet this enhanced demand rice production is anticipated to annually increase in the range of 8–10 million tons over the next 20 years (Liu *et al*., 2013). Furthermore, the increasing demand on water in municipal and industrial sectors and increasing climatic variability necessitates revision of rice production in the context of future changes in the accessibility to water resources. Consequently, it is predicted that rice farmers will face economic water scarcity as result of increasing costs for irrigation and physical water scarcity as supplies for irrigation shrink (Bouman *et al*., 2005, 2007). Especially Asia, harboring 89% of the world's rice paddies (FAOSTAT2012), will be heavily influenced by water scarcity (Tuong and Bouman 2003). For that reason, Asian rice farmers will be coerced to decrease their water consumption during irrigation of rice fields in times of low water availability (e.g. dry season).

Several management strategies have been developed to reduce water requirements of wetland rice fields such as alternate wetting and drying (Wassmann *et al*., 2000a, b), mid-season drainage (Wassmann *et al*., 2000b), intermittent drainage (Yagi *et al*., 1996) or system of rice intensification (Stoop *et al*., 2002). These methods are all based on restricted irrigation patterns under cultivation of flooded rice. Thereby, short periods of drainage allow

the regeneration of inorganic electron acceptors and will affect the processes involved in anaerobic degradation of organic matter.

However, a different approach to reduce water consumption from rice fields is to rotate rice cultivation with upland crops that need less water such as maize. In this manner innate irrigated rice fields will face long periods (several months) of drainage along with upland field conditions. This includes long-term aeration of the soil possibly causing oxygen stress for inhabiting soil microorganisms. Indeed, a change from traditional rice–rice (wet–dry season) systems to rice–maize cropping systems is observable across tropical and subtropical Asia. This is initiated not only by the water scarcity but also by increasing demand of maize for food (poultry) and biofuel production (Weller *et al*., 2015). Accordingly rice–maize systems are notably implemented today (Timsina *et al*., 2010). Changes from rice cultivation (flooded soil) to water-saving practices or diversified cropping systems were shown to impact yields, soil carbon and nitrogen turnover (Bronson *et al*., 1997a, b; Abao *et al*., 2000; Wassmann *et al*., 2000a) along with greenhouse gases emissions (Nishimura *et al*., 2005, 2011; Weller *et al*., 2015).

The shift between flooded and drained conditions, linked with completely different redox conditions, will also affect the activity and composition of the present microbial communities. In drained upland soils CO_2 is the sole mineralization product whereas only low CH_4 formation was reported (Dutar and Verchot, 2007; Soussana *et al*., 2007). Drained uplands CH_4 production can only occur in anoxic micro-niches and is generally of small extent (Megongial and Guenther, 2008). However, it has been shown that the likelihood of drained rice field soils to be sinks for atmospheric CH_4 is rather small (Jäckel *et al*., 2001).

In general, the bacterial community composition is prone to temporal and spatial changes as a result of changing soil conditions in rice field soil (Asakawa and Kimura, 2008; Noll *et al*., 2005; Shrestha *et al*., 2007; Shrestha *et al*., 2009). For instance, water-saving practices have been shown to impact the bacterial community abundance and composition in rice field soil under field conditions (Ahn *et al*., 2014; Itoh *et al*., 2013). Furthermore, as seen in the previous chapter, the root system of plants can impact the soil microbial community. Accordingly, crop rotational systems may lead to changes in bacterial community as each plant species possesses individual root exudates, which were already

shown to impact soil bacterial community structure (Marschner *et al*., 2001; Haichar *et al*., 2008). The bacterial abundance, diversity and community composition was impacted by upland-upland crop rotations (Yin *et al*., 2010; Acosta-Martinez *et al*., 2008) and rotations of flooded rice with upland crops such as mungbean, maize, alfalfa (Xuan *et al*., 2012; Lopes *et al*., 2014). However, in a few cases crop rotations caused only minor effects on the bacterial community (Fernandez Scavino *et al*., 2013; Zhao *et al*., 2014).

The composition of archaeal communities in rice field soil seems to be relatively stable even when the soil conditions are changed (Krüger *et al*., 2005; Watanabe *et al*., 2007). In contrast, the activity of archaeal communities changes (Krüger *et al*., 2001; Krüger *et al*., 2005; Watanabe *et al*., 2007). Furthermore, crop rotations with upland crops were shown to affect archaeal communities only minor (Asakawa and Hayano, 1995; Watanabe *et al*., 2006, 2011; Fernandez Scavino *et al*., 2013). The archaeal community structure remained stable even under upland field conditions for up to seven months. Nevertheless, several laboratory experiments demonstrated that drainage and oxygen exposure can impair the growth of methanogenic archaea (Ma and Lu, 2001; Ma *et al*., 2012; Yuan *et al*., 2009). Accordingly, in a field study a moderate influence on the methanogenic archaeal community as effect of water management was observed (Watanabe *et al*., 2013). Recently, decreased abundance of methanogenic archaea along with changes in community composition was reported in a flooded rice-soybean crop rotation (Liu *et al*., 2015). Nevertheless, the knowledge concerning the impact of crop rotations on the microbial community in rice field soil is rather limited and has to be improved in order to face increasing water demands and anticipated water scarcity.

1.6 Aims of the study

Chapter 2: The influence of rice plants on the microbial community structure in flooded paddy soil at different growth stages

Plants are known to be shaping the microbial community in the rhizosphere by providing organic and inorganic compounds via their roots, a process called rhizodeposition. For rice plants it was shown that the quantity and quality of rhizodeposits varies with plant age. *Does the rice plant shape the microbial community in the rice rhizosphere? Do changes occur in the structure of the microbial community with changing rice plant growth stages?*

Chapter 3: Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage

The rice plant is influencing the microbial community in the soil by providing organic and inorganic compounds via the root system – a dynamic process. Drainage and maize cultivation under upland conditions will result in completely different redox conditions. *Is the microbial community in rice field soil impacted by rice plant growth stage under field conditions? Does the rice specific microbial community react to non-flooded conditions and to the presence or absence of maize plants?*

Chapter 4: Crop rotation of flooded rice with upland maize impacts resident and active methanogenic community in rice field soil

Crop rotation system between flooded rice and upland maize will lead to dramatic changes in field conditions like completely different redox conditions. Additionally, the plant type may have an effect on the community structure of soil microbes. *Does crop rotation with upland maize lead to changes in the microbial community in the soil? Do these communities recover during re-flooding and paddy rice cultivation? Do long term effects occur?*

1.7 References

Abao, E.B., Bronson, K.F., Wassmann, R., and Singh, U. (2000). Simultaneous records of methane and nitrous oxide emissions in rice-based cropping systems under rainfed conditions. *Nutr. Cycl. Agroecosyst.* 58, 131-139. doi:10.1023/A:1009842502608.

Acosta-Martinez, V., Dowd, S., Sun, Y., and Allen, V. (2008). Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. *Soil Biol. Biochem*. 40, 2762-2770.

Ahn, J. H., Choi, M. Y., Kim, B. Y., Lee, J. S., Song, J., Kim, G. Y., *et al*. (2014). Effects of water-saving irrigation on emissions of greenhouse gases and prokaryotic communities in rice paddy soil. *Microb. Ecol.* 68, 271-283. doi: 10.1007/s00248-014-0371-z

Angel, R., Matthies, D., and Conrad, R. (2011). Activation of methanogenesis in arid biological soil crusts despite the presence of oxygen. *PLoS ONE* 6:e20453. doi:10.1371/journal.pone.0020453.

Angel, R., Claus, P., and Conrad, R. (2012). Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *ISME J.* 6, 847-862.

Armstrong, W. (1979) Aeration in higher plants. *Adv. Bot. Res.* 7, 226-332.

Asakawa, S. and Hayano, K. (1995). Population of methanogenic bacteria in paddy field soil under double cropping conditions (rice-wheat). *Biol. Fertil. Soils* 20, 113-117.

Asakawa, S. and Kimura, M. (2008). Comparison of bacterial community structures at main habitats in paddy field ecosystem based on DGGE analysis [review]. *Soil Biol. Biochem.* 40, 1322-1329.

Aschenbach, K., Conrad, R., Řeháková, K., Doležal, J., Janatková, K., and Angel, R. (2013). Methanogens at the top of the world: occurrence and potential activity of methanogens in newly deglaciated soils in high-altitude cold deserts in the Western Himalayas. *Front. Microbio*. 4, 359. doi:10.3389/fmicb.2013.00359.

Aulakh, M. S., Wassmann, R., Bueno, C., Kreuzwieser, J., and Rennenberg, H. (2001). Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biol*. 3, 139-148. doi: 10.1055/s-2001-12905.

Aydin, M., Verhulst, K. R., Saltzman, E. S., Battle, M. O., Montzka, S. A., Blake, D. R., *et al*. (2011). Recent decreases in fossil-fuel emissions of ethane and methane derived from firn air. *Nature* 476, 198-201.

Badri, D.V., and Vivanco, J.M., (2009). Regulation and function of root exudates. *Plant Cell Environ*. 32, 666-681.

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., and Vivanco, J. M., (2004). How plants communicate using the underground information superhighway. *Trends Plant Sci*. 9, 26-32.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M., (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol*. 57, 233-266.

Barber, D. A. and Martin, J. K. (1976). The release of organic substances by cereal roots into soil. *New Phytol.* 76, 69-80.

Bertin, C., Yang, X., and Weston, L.A., (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256, 67-83.

Boetius, A., Ravenschlag, K., Schubert, C. J., Rickert, D.,Widdel, F., Gieseke, A.,*et al*. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407, 623-626.

Borrel, G., Parisot, N., Harris, H. M., Peyretaillade, E., Gaci, N., Tottey, W., *et al*. (2014). Comparative genomics highlights the unique biology of *Methanomassiliicoccales*, a *Thermoplasmatales*-related seventh order of methanogenic archaea that encodes pyrrolysine. *BMC genomics* 15, 679.

Bosse, U., and Frenzel, P. (1997). Activity and distribution of methane-oxidizing bacteria in flooded rice soil microcosms and in rice plants (*Oryza sativa*). *Appl. Environ. Microbiol*. 63, 1199-1207.

Bouman, B. A. M. (2007). A conceptual framework for the improvement of crop water productivity at different spatial scales. *Agric. Syst*. 93, 43-60.

Bouman, B. A. M., Hengsdijk, H., Hardy, B., Bindraban, P. S., Tuong, T. P., and Ladha, J. K. (2002) (Eds.): "Water-wise rice production", in: *Proceedings of the International Workshop on Water-wise Rice Production 8–11 April 2002*, International Rice Research Institute, Los Baños, Philippines.

Bouman, B. A. M., Humphreys, E., Tuong, T. P., and Barker, R. (2007). Rice and water. *Adv. Agron*. 92, 187-237.

Bouman, B. A. M., Peng, S., Castaneda, A. R., and Visperas, R. M. (2005). Yield and water use of irrigated tropical aerobic rice systems. *Agric. Water Manag*, 74, 87-105.

Bousquet, P., Ciais, P., Miller, J. B., Dlugokencky, E. J., Hauglustaine, D. A., Prigent, C., *et al*. (2006). Contribution of anthropogenic and natural sources to atmospheric methane variability. *Nature* 443, 439-443.

Bronson, K. F., Neue, H. U., Singh, U., and Abao, E. B. (1997a). Automated chamber measurements of methane and nitrous oxide flux in a flooded rice soil. 1. Residue, nitrogen, and water management. *Soil Sci. Soc. Am. J*. 61,981-987.

Bronson, K. F., Singh, U., Neue, H. U., and Abao, E. B. (1997b). Automated chamber measurements of methane and nitrous oxide flux in a flooded rice soil. 2. Fallow period emissions. *Soil Sci. Soc. Am. J.* 61, 988-993.

Caldwell, S. L., Laidler, J. R., Brewer, E. A., Eberly, J. O., Sandborgh, S. C., and Colwell, F. S. (2008). Anaerobic oxidation of methane: Mechanisms, bioenergetics, and the ecology of associated microorganisms. *Environ. Sci. Technol.* 42, 6791-6799.

Chin, K.-J., Rainey, F. A., Janssen, P. H. and Conrad, R. (1998). Methanogenic degradation of polysaccharides and the characterization of polysaccharolytic clostridia from anoxic rice field soil. *Syst. Appl. Microbiol.* 21, 185-200.

Conrad, R. (2007). Microbial ecology of methanogens and methanotrophs. *Adv. Agron*. 96, 1-63.

Conrad, R., and Frenzel, P. (2002). "Flooded soils". in: *Encyclopedia of Environmental Microbiology*., ed. G. Britton, John Wiley & Sons, New York, USA, 1316-1333. doi:10.1002/0471263397.env034

Conrad, R., Klose, M., Lu, Y., and Chidthaisong, A. (2012). Methanogenic pathway and archaeal communities in three different anoxic soils amended with rice straw and maize straw. *Front Microbiol* 3, 4. doi:10.3389/fmicb.2012.00004.

Cowardin, L. M., Carter, V., and Golet, F. C. (1979). "Classification of wetlands and deepwater habitats of the united states", in: *Fish and Wildlife Service*, US Department of the Interior Washington, DC, USA.

Denman, K., Brasseur, G., Chidthaisong, A., Clais, P., Cox, R., Dickinson, D., *et al*. (2007). "Couplings between changes in the climate system and biogeochemistry", in: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., *et al*., Cambridge University Press: Cambridge, United Kingdom and New York, New York, USA, 541-584.

Dennis, P. G., Miller, A. J., and Hirsch, P. R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities?. *FEMS Microbiol. Ecol.* 72, 313-327.

Derrien, D., Marol, C., and Balesdent, J. (2004). The dynamics of neutral sugars in the rhizosphere of wheat: an approach by ^{13}C pulse-labelling and GC/C/IRMS. *Plant Soil* 267, 243-253.

Dlugokencky, E. J., Bruhwiler, L., White, J. W. C., Emmons, L. K., Novelli, P. C., Montzka, S. A., *.et al*. (2009). Observational constraints on recent increases in the atmospheric CH_4 burden. *Geophys. Res. Lett*. 36, L18803, doi:10.1029/2009GL039780.

Dlugokencky, E. J., Masarie, K. A., Lang, P. M., and Tans, P. P. (1998). Continuing decline in the growth rate of the atmospheric methane burden. *Nature* 393, 447-450.

Dutaur, L., and Verchot, L. V. (2007). A global inventory of the soil CH_4 sink. *Global Biogeochem.Cy*. 21, GB4013, doi:10.1029/2006GB002734.

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., *et al*. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci.* 112, E911-E920.

Eller, G., and Frenzel, P. (2001). Changes in activity and community structure of methane-oxidizing bacteria over the growth period of rice. *Appl. Environ. Microbiol*. 67, 2395-2403.

Erkel, C., Kube, M., Reinhardt, R., and Liesack, W. (2006). Genome of Rice Cluster I archaea—the key methane producers in the rice rhizosphere. *Science*, 313, 370-372.

Ettwig, K. F., Shima, S., van de Pas-Schoonen, K. T., Kahnt, J., Medema, M. H., Op den Camp, H. J. M., *et al*. (2008). Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environ. Microbiol.* 10, 3164-3173.

Ettwig, K. F., Butler, M. K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M. M., *et al*. (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 464, 543-548.

FAOSTAT (2012) Food and Agricultural Organization of the United Nations, Available online at: http://faostat.fao.org/site/291/default.aspx, access date 24.11.2014.

Fernandez Scavino, A., Ji, Y., Pump, J., Klose, M., Claus, P., and Conrad, R. (2013). Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated rice fields. *Environ. Microbiol.* 15, 2588-2602. doi: 10.1111/1462-2920.12161

Fetzer, S., Bak, F., and Conrad, R. (1993). Sensitivity of methanogenic bacteria from paddy soil to oxygen and desiccation. *FEMS Microbiol. Ecol.* 12, 107-115.

Fetzer, S., and Conrad, R. (1993). Effect of redox potential on methanogenesis by *Methanosarcina barkeri*. *Arch. Microbiol.* 160, 108-113.

Frenzel, P., Rothfuss, F., and Conrad, R. (1992). Oxygen profiles and methane turnover in a flooded rice microcosm. *Biol. Fertil. Soils* 14, 84-89.

Friedrich, M. W. (2005). Methyl-coenzyme M reductase genes: unique functional markers for methanogenic and anaerobic methane-oxidizing archaea. *Methods Enzymol*. 397, 428-442.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D., *et al*. (2007). "Changes in atmospheric constituents and in radiative forcing". in: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., *et al*., Cambridge University Press: Cambridge, United Kingdom and New York, New York, USA, 130-234.

Gilbert, B., and Frenzel, P. (1995). Methanotrophic bacteria in the rhizosphere of rice microcosms and their effect on porewater methane concentration and methane emission. *Biol. Fertil. Soils* 20, 93-100.

Grosse, W., and Bauch, C. (1991). Gas transfer in floating-leaved plants. *Vegetatio*, 97, 185-192.

Großkopf, R., Janssen, P. H., and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64, 960-969.

Haichar, Z. F., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., *et al*. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME J.* 2, 1221-1230.

Haichar, Z. F., Santaella, C., Heulin, T., and Achouak, W. (2014). Root exudates mediated interactions belowground. *Soil. Biol. Biochem.* 77, 69-80.

Haroon, M. F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., *et al*. (2013). Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 500, 567-570.

Harrison, B. K., Zhang, H., Berelson, W., and Orphan, V. J. (2009). Variations in archaeal and bacterial diversity associated with the sulfate-methane transition zone in continental margin sediments (Santa Barbara Basin, California). *Appl. Environ. Microbiol.* 75, 1487-1499.

Hartmann, A., Schmid, M., van Tuinen, D., and Berg, G. (2009). Plant-driven selection of microbes. *Plant Soil* 321, 235-257.

Hartmann, D. L., Klein Tank, A. M. G., Rusticucci, M., Alexander, L.V., Brönnimann, S., Charabi, Y., *et al*. (2013). "Observations: Atmosphere and surface", in: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change,* eds. Stocker, T. F., Qin, D., Plattner, G.-K. Tigor, M., Allen, S. K. Boschung, J., Naules, A., Xia, Y., Bex, V., and Midgley, P.M., Cambridge University Press, Cambridge, United Kingdom and New York, New York, USA, 159-254.

Hawes, M. C., Gunawardena, U., Miyasaka, S., and Zhao, X. (2000). The role of root border cells in plant defense. *Trends Plant Sci*. 5, 128-133.

Hedderich, R., and Whitman, W. (2006). "Physiology and biochemistry of the methane-producing Archaea", in: *The Prokaryotes, Vol. 2, 3rd,* ed. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt, *et al*., New York: Springer Verlag, 1050-1079.

Hernández, M., Dumont, M. G., Yuan, Q., and Conrad, R. (2015). Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. *Appl. Environ. Microbiol.* doi:10.1128/AEM.03209-14

Hiltner, L. (1904). Über neuere Erfahrungen und Problem auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brachte. *Arb. Dt. Landwges.* 98, 59-78.

Hu, B. L., Shen, L. D., Lian, X., Zhu, Q., Liu, S., Huang, Q., *et al*. (2014). Evidence for nitrite-dependent anaerobic methane oxidation as a previously overlooked microbial methane sink in wetlands. *Proc. Natl. Acad. Sci.* 111, 4495-4500.

Hutsch, B.W., Augustin, J., and Merbach, W. (2000). Plant rhizodeposition an important source for carbon turnover in soils. *J. Plant Nutr. Soil Sci.* 165, 397-407.

Itoh, H., Ishii, S., Shiratori, Y., Oshima, K., Otsuka, S., Hattori, M., and Senoo, K. (2013). Seasonal transition of active bacterial and archaeal communities in relation to water management in paddy soils. *Microbes Environ.* 28, 370-380.

Jäckel, U., Schnell, S., and Conrad, R. (2001). Effect of moisture, texture and aggregate size of paddy soil on production and consumption of CH_4. *Soil. Biol. Biochem.* 33, 965-971.

Jarrell, K. F. (1985). Extreme oxygen sensitivity in methanogenic archaebacteria. *Bioscience* 35, 298-302.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323-329.

Jones, D. L., Hodge, A., and Kuzyakov, Y. (2004). Plant and mycorrhizal regulation of rhizodeposition. *New Phytol*. 163, 459-480.

Jones, D., Nguyen, C., and Finlay, D.R. (2009). Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil* 321, 5-33.

Kai, F. M., Tyler, S. C., Randerson, J. T., and Blake, D. R. (2011). Reduced methane growth rate explained by decreased Northern Hemisphere microbial sources. *Nature*, 476, 194-197.

Keppler, F., Hamilton, J. T. G., Braß, M., and Rockmann, T. (2006). Methane emissions from terrestrial plants under aerobic conditions. *Nature* 439,187-191.

Krüger, M., Frenzel, P., and Conrad, R. (2001). Microbial processes influencing methane emission from rice fields. *Global Change Biol*. 7, 49-63.

Krüger, M., Frenzel, P., Kemnitz, D. and Conrad, R. (2005). Activity, structure and dynamics of the methanogenic archaeal community in a flooded Italian rice field. *FEMS Microbiol. Ecol*. 51, 323-33.

Liesack, W., Schnell, S., and Revsbech, N. P. (2000). Microbiology of flooded rice paddies. *FEMS Microbiol. Rev*. 24, 625-645.

Liu, D., Ishikawa, H., Nishida, M., Tsuchiya, K., Takahashi, T., Kimura, M., and Asakawa, S. (2015). Effect of paddy-upland rotation on methanogenic archaeal community structure in paddy field soil. *Microb. Ecol*. 69, 160-168.

Liu, M., Lin, S., Dannenmann, M., Tao, Y., Saiz, G., Zuo, *et al*. (2013). Do water-saving ground cover rice production systems increase grain yields at regional scales? *Field Crop. Res*. 150, 19-28.

Liu, Y., and Whitman, W. B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.* 1125, 171-189.

Lopes, A. R., Manaia, C. M., and Nunes, O. C. (2014). Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. *FEMS Microbiol. Ecol.* 87, 650-663.

Loulergue, L., Schilt, A., Spahni, R., Masson-Delmotte, V., Blunier, T., Lemieux, B., *et al*. (2008). Orbital and millennial-scale features of atmospheric CH_4 over the past 800,000 years. *Nature* 453, 383-386.

Lowe, D. C. (2006). A green source of surprise. *Nature* 439, 148-149.

Lu, Y., and Conrad, R. (2005). In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309, 1088-1090.

Luton, P. E., Wayne, J. M., Sharp, R. J., and Riley, P. W. (2002). The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* 148, 3521-3530.

Lynch, J. M., and Whipps, J. M. (1990). Substrate flow in the rhizosphere. *Plant Soil* 129, 1-10. http://dx.doi.org/10.1007/BF00011685.

Ma, K., and Lu, Y. (2011). Regulation of microbial methane production and oxidation by intermittent drainage in rice field soil. *FEMS Microbiol. Ecol.* 75, 446-456.

Ma, K., Conrad, R., and Lu, Y. (2012). Responses of methanogen *mcrA* genes and their transcripts to an alternate dry/wet cycle of paddy field soil. *Appl. Environ. Microbiol.* 78, 445-454.

Maclean, J. L., Dawe, D. C., Hardy, B., and Hettel, G. P. (eds) (2002). "Rice almanac: source book for the most important economic activity on earth, 3rd edn" CABI Publishing, Wallingford, United Kingdom.

Marschner, P., Yang, C. H., Lieberei, R., and Crowley. D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol. Biochem.* 33, 1437-1445.

McLeod, A. R., Fry, S. C., Loake, G. J., Messenger, D. J., Reay, D. S., Smith, K. A., *et al*. (2008). Ultraviolet radiation drives methane emissions from terrestrial plant pectins. *New Phytol.* 180, 124-132.

Megonigal, J. P., and Guenther, A. B. (2008). Methane emissions from upland forest soils and vegetation. *Tree Physiol.* 28, 491-498.

Narihiro, T., and Sekiguchi, Y. (2011). Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea. *Microb. Biotech.* 4, 585-602.

Neue, H. U., and Roger, P. A. (1993). "Rice agriculture: factors controlling emissions", in: *Atmospheric Methane: Sources, Sinks, and Role in Global Change*, Springer Berlin Heidelberg, Germany, 254-298.

Nguyen, C. (2003). Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* 23, 375-396.

Nicol, G. W., Glover, L. A., and Prosser, J. I. (2003). The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. *Environ. Microbiol*. 5, 152-162.

Nishimura, S., Sawamoto, T., Akiyama, H., Sudo, S., Cheng, W., and Yagi, K. (2005). Continuous, automated nitrous oxide measurements from paddy soils converted to upland crops. *Soil Sci. Soc. Am. J.* 69, 1977-1986. doi:10.2136/sssaj2005.0035.

Nishimura, S., Akiyama, H., Sudo, S., Fumoto, T., Cheng, W., and Yagi, K. (2011). Combined emission of CH_4 and N_2O from a paddy field was reduced by preceding upland crop cultivation. *Soil Sci. Plant Nutr*. 57, 167-178.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ.Microbiol.* 7, 382-395.

Patrick, W. H. Jr., and Reddy, C. N. (1978). "Chemical changes in rice soils", in: *International Rice Research Institute (ed.), Soils and rice*, IRRI, Los Banos, Philippines, 361-379.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van der Putten, W.H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789-799.

Ponnamperuma, F. N. (1972). The chemistry of submerged soils. *Adv.Agron*. 24, 29-96.

Poplawski, A. B., Martensson, L., Wartiainen, I., and Rasmussen, U. (2007). Archaeal diversity and community structure in a Swedish barley field: specificity of the Ek510r/ (EURY498) 16S rDNA primer. *J. Microbiol. Methods* 69, 161-173.

Pump, J., Pratscher, J., and Conrad, R. (2014). Colonization of rice roots with methanogenic archaea controls photosynthesis-derived CH_4 emission. *Environ. Microbiol. Rep.* doi:10.1111/1462-2920.12675.

Raghoebarsing, A. A., Pol, A., van de Pas-Schoonen, K. T., Smolders, A. J. P., Ettwig, K. F., Rijpstra, W. I. C., *et al*. (2006). A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* 440, 918-921.

Ramakrishnan, B., Lueders, T., Dunfield, P. F., Conrad, R., and Friedrich, M. W. (2001). Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production. *FEMS Microbiol. Ecol.* 37, 175-186.

Read, D. B., Bengough, A. G., Gregory, P. J., Crawford, J. W., Robinson, D., Scrimgeour, C. M., *et al*. (2003). Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil. *New Phytol.* 157, 315-321.

Rigby, M., Prinn, R. G., Fraser, P. J., Simmonds, P. G., Langenfelds, R. L., Huang, J., *et al*. (2008). Renewed growth of atmospheric methane. *Geophys. Res. Lett*. 35, L22805. doi:10.1029/2008GL036037.

Ryan, P.R., and Delhaize, E., (2001). Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Mol. Biol*. 52, 527-560.

Shrestha, P. M., Kube, M., Reinhardt, R., and Liesack, W. (2009). Transcriptional activity of paddy soil bacterial communities. *Environ. Microbiol.* 11, 960-970.

Shrestha, P. M., Noll, M., and Liesack, W. (2007). Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. *Environ. Microbiol*. 9, 2464-2474.

Soussana, J. F., Allard, V., Pilegaard, K., Ambus, P., Amman, C., Campbell, C., *et al*. (2007). Full accounting of the greenhouse gas (CO_2, N_2O, CH_4) budget of nine European grassland sites. *Agric. Ecosyst. Environ.* 121, 121-134.

Spahni, R., Chappellaz, J., Stocker, T. F., Loulergue, L., Hausammann, G., Kawamura, K., *et al*. (2005). Atmospheric methane and nitrous oxide of the late Pleistocene from Antarctic ice cores. *Science* 310, 1317-1321.

Stoop, W. A., Uphoff, N., and Kassam, A. (2002). A review of agricultural research issues raised by the system of rice intensification (SRI) from Madagascar: opportunities for improving farming systems for resource-poor farmers. *Agric. Sys*. 71, 249-274.

Stubbs, V.E., Standing, D., Knox, O.G., Killham, K., Bengough, A.G., and Griffiths, B. (2004). Root border cells take up and release glucose-C. *Ann. Bot. Lond*. 93, 221-224.

Thauer, R. K. (1998). Biochemistry of methanogenesis: A tribute to Marjory Stephenson: 1998 Marjory Stephenson Prize Lecture. *Microbiology* 144, 2377-2406.

Thauer, R. K. (2011). Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO_2. *Curr. Opin. Microbiol.* 14, 292-299.

Timsina, J., Jat, M. L., and Majumdar, K. (2010). Rice-maize systems of South Asia: current status, future prospects and research priorities for nutrient management. *Plant Soil* 335, 65-82.

Tuong, T. P., and Bouman, B. A. M. (2003). "Rice production in water-scarce environments". in: *Water productivity in agriculture: limits and opportunities for improvement*, eds. Kijne, J. W., Barker, R., Molden, D., CABI Publishing, Wallingford, United Kingdom, 53-67.

Tuong, T. P., Bouman, B. A. M., and Mortimer, M. (2005). More rice, less water-integrated approaches for increasing water productivity in irrigated rice-based systems in Asia. *Plant Prod. Sci.* 8, 231-241.

Ueki, A., Ono, K., Tsuchiya, A., and Ueki, K. (1997). Survival of methanogens in air-dried paddy field soil and their heat tolerance. *Water Sci. Tech.* 36, 517-522.

Valentine, D. L. (2007). Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat. Rev. Microbiol.* 5, 316-323.

Van Nguyen, N., and Ferrero, A. (2006). Meeting the challenges of global rice production. *Paddy Water Environ*. 4, 1-9. doi: 10.1007/s10333-005-0031-5.

Wassmann, R., Buendia, L. V., Lantin, R. S., Bueno, C. S., Lubigan, L. A., Umali, A., *et al* (2000a). Mechanisms of crop management impact on methane emissions from rice fields in Los Banos, Philippines. *Nutr Cycl Agroecosyst.* 58, 107-119. doi:10.1023/A:1009838401699.

Wassmann, R., Lantin, R. S., Neue, H. U., Buendia, L. V., Corton, T. M., and Lu, Y. (2000b) Characterization of methane emissions from rice fields in Asia. III. Mitigation options and future research needs. *Nutr Cycl Agroecosyst.* 58, 23-36. doi:10.1023/A:1009874014903.

Watanabe, T., Hosen, Y., Agbisit, R., Llorca, L., Katayanagi, N., Asakawa, S., and Kimura, M. (2013). Changes in community structure of methanogenic archaea brought about by water-saving practice in paddy field soil. *Soil Biol. Biochem.* 58, 235-243.

Watanabe, T., Kimura, M., and Asakawa, S. (2006). Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). *Soil Biol. Biochem*. 38, 1264-1274. doi:10.1016/j.soilbio.2005.09.020.

Watanabe, T., Kimura, M. and Asakawa, S. (2007). Dynamics of methanogenic archaeal communities based on rRNA analysis and their relation to methanogenic activity in Japanese paddy field soils. *Soil Biol. Biochem.* 39, 2877-2887.

Watanabe, A., Takeda, T., and Kimura, M. (1999). Evaluation of origins of CH_4 carbon emitted from rice paddies. *J. Geophys. Res.* 104, 23623-23629.

Watanabe, T., Wang, G., Lee, C. G., Murase, J., Asakawa, S., and Kimura, M. (2011). Assimilation of glucose-derived carbon into methanogenic archaea in soil under unflooded condition. *Appl. Soil Ecol*. 48, 201-209. doi:10.1016/j.apsoil.2011.03.005.

Weller, S., Kraus, D., Ayag, K. R. P., Wassmann, R., Alberto, M. C. R., Butterbach-Bahl, K., and Kiese, R. (2015). Methane and nitrous oxide emissions from rice and maize production in diversified rice cropping systems. *Nutr. Cycl. Agroecosyst*. 101, 37-53.

Whitman, W. B., Bowen, T. L., and Boone, D. R. (2006). "The methanogenic bacteria", in: *The prokaryotes*, Springer New York, New York, USA, 165-207.

Woese, C. R., and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci.* 74, 5088-5090.

Wu, X. L., Friedrich, M. W., and Conrad, R. (2006). Diversity and ubiquity of thermophilic methanogenic archaea in temperate anoxic soils. *Environ. Microbiol.* 8, 394-404.

Xuan, D. T., Guong, V. T., Rosling, A., Alström, S., Chai, B., and Högberg, N. (2012). Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biol. Fertil. Soils* 48, 217-225.

Yagi, K., Tsuruta, H., Kanda, K., and Minami, K. (1996). Effect of water management on methane emission from a Japanese rice paddy field: auto-mated methane monitoring. *Glob. Biogeochem. Cycles* 10, 255-267. doi:10.1029/96GB00517

Yao, H., Conrad, R., Wassmann, R., and Neue, H. U. (1999). Effect of soil characteristics on sequential reduction and methane production in sixteen rice paddy soils from China, the Philippines, and Italy. *Biogeochemistry* 47, 269-295.

Yin, C., Jones, K. L., Peterson, D. E., Garrett, K. A., Hulbert, S. H., and Paulitz, T. C. (2010). Members of soil bacterial communities sensitive to tillage and crop rotation. *Soil Biol. Biochem*. 42, 2111-2118.

Yuan, Y., Conrad, R., Lu, Y. (2009). Responses of methanogenic archaeal community to oxygen exposure in rice field soil. *Environ. Microbiol. Rep.* 1, 347-354.

Zhao, J., Zhang, R., Xue, C., Xun, W., Sun, L., Xu, Y., and Shen, Q. (2014). Pyrosequencing reveals contrasting soil bacterial diversity and community structure of two main winter wheat cropping systems in China. *Microb. Ecol*. 67, 443-453.

Zhou, L., Wang, Y., Long, X. E., Guo, J., and Zhu, G. (2014). High abundance and diversity of nitrite-dependent anaerobic methane-oxidizing bacteria in a paddy field profile. *FEMS Microbiol. Ecol.* 360, 33-41.

Zhu, G., Jetten, M. S. M., Kuschk, P., Ettwig, K. F., and Yin, C. (2010). Potential roles of anaerobic ammonium and methane oxidation in the nitrogen cycle of wetland ecosystems. *Appl. Microbiol. Biotechnol.* 86, 1043-1055.

Zhu, G., Zhou, L., Wang, Y., Wang, S., Guo, J., Long, X. E., *et al*. (2014). Biogeographical distribution of denitrifying anaerobic methane oxidizing bacteria in Chinese wetland ecosystems. *Environ. Microbiol. Rep.* 7, 128-138.

Zinder, S. H. (1993). "Physiological ecology of methanogens", in: *Methanogenesis*, Springer, New York, USA, 128-206.

Chapter 2

The influence of rice plants on the microbial community structure in flooded paddy soil at different growth stages[#]

Björn Breidenbach[1], Judith Pump[1], Marc G. Dumont[1]

1. Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Straße 10, 35043

Marburg, Germany

Contributions:

B.B. designed the study, performed the greenhouse experiment, performed all lab work (nucleic-acid extractions, T-RFLP analysis, qPCR analysis, PCRs for 454 pyrosequencing analysis), performed statistical analysis, evaluated the data and wrote the manuscript.

J.P. designed the study, performed greenhouse experiment, evaluated the data and performed statistical analysis.

M.G.D. designed the study, performed data evaluation and statistically analysis, wrote the manuscript.

Parts of this work have been obtained during the master thesis of Björn Breidenbach: Einfluss der Wachstumsstadien der Reispflanze auf die mikrobielle Gemeinschaft im Reisfeldboden.

2.1 Abstract

The microbial community in the rhizosphere environment is critical for the growth of land plants and the recycling of soil organic matter. The objective of this study was to determine the extent to which rice plants shape the microbial community in rice field soil over the course of a growing season. Rice (*Oryza sativa*) was cultivated under greenhouse conditions in rice field soil from Vercelli, Italy and the microbial community in the rhizosphere of planted soil microcosms was characterized at four plant growth stages using quantitative PCR and 16S rRNA gene pyrotag sequencing. The abundance of 16S rRNA genes were on average twice that of unplanted soil, indicating that microbial growth was stimulated in the rice rhizosphere. The 16S rRNA sequence analysis could distinguish the microbial community in planted with unplanted soil, but the overall difference was relatively small. Approximately 2% of operational taxonomic units (97% 16S rRNA identity) could be identified as significantly higher in either planted or unplanted soil. There was only weak evidence for a temporal pattern in soil community compositions. The conclusion is there was not a major shift in the relative abundance of microbial groups in planted soil despite a higher absolute abundance, suggesting that the soil microbial community is highly adapted to rice plants and that the microbial community in Vercelli rice field soil is relatively stable.

2.2 Introduction

Plants depend on their root system for the uptake of nutrients and water from the soil. The root system further allows plants to influence the microbes inhabiting the soil. The zone in the soil surrounding living roots and influenced by plant activity was first defined by Hiltner (1904) and termed the rhizosphere. The rhizosphere is characterized by highly dynamic interactions between plants and the soil-borne organisms including archaea, bacteria, fungi, nematodes and insects (Ryan and Delhaize, 2001; Bais *et al*., 2004). Permanent competition for space, water, minerals and macronutrients give rise to interactions between soil microbes and plants. These include symbiotic interactions with growth promoting bacteria, or potential attack from pathogens (Bais *et al*., 2006; Haichar *et al*., 2014; Philippot *et al*., 2013).

Plants influence the microbial community in soil by root-mediated release of organic and inorganic compounds, termed rhizodeposition. Rhizodeposition can be divided in three categories: (I) the release of low- and high-molecular weight compounds via the roots (root exudates), (II) a gelatinous layer surrounding root tips (mucilage) and (III) metabolically active root cells, programmed to be released from the root into the surrounding soil (border cells) (Badri and Vivanco, 2009; Hawes *et al*., 2000; Stubbs *et al*., 2004; Jones *et al*., 2009; Read *et al*., 2003). The amount of root exudation may vary since the quality and quantity is a function of plant species, plant age as well as of external biotic and abiotic factors (Jones *et al*., 2004). Root exudation by rice plants has been shown to vary in composition and rate during plant development (Aulakh *et al*., 2001).

Rhizodeposits are important substrates for the soil microbes since up to 90% is metabolized by the root-associated microorganisms (Lynch and Whip; 1990). Rice cultivation is also a major source of atmospheric methane (CH_4), of which ~60% originates from root exudates or dead root material (Watanabe *et al*., 1999). Pulse-labeling experiments have identified methanogenic archaea incorporating plant-derived carbon in the rice rhizosphere (Lu and Conrad, 2005; Zhu *et al*., 2014). The microbial communities in the endosphere (root interior), the rhizoplane (root surface) and the rhizosphere (Edwards *et al*., 2015) have been described and the bacterial lineages metabolizing plant-derived carbon in

the root environment have been identified (Hernández *et al*., 2015). The rice phyllosphere microbial community has been characterized by 16S rRNA pyrotag sequencing (Ren *et al*., 2014) as well as using metagenomic and proteomic approaches (Knief *et al*. 2012).

Despite the relatively detailed knowledge of the microbial community associated with rice plants, it is not known to what extent rice plants shape the rhizosphere microbial community during the course of plant growth. To investigate this, we performed a greenhouse experiment comparing planted and unplanted Vercelli rice field soil over a full vegetation period. In total four time points (34, 52, 62 and 90 days after planting) covering all plant growth stages were monitored. We quantified the absolute abundance of Archaea and Bacteria by quantitative PCR (qPCR) targeting the 16S rRNA gene. The microbial community composition in rhizosphere or bulk (unplanted) soil was evaluated by pyrotag sequencing analysis of the 16S rRNA gene.

2.3 Materials and methods

2.3.1 Microcosms and incubations

Soil was sampled from rice fields at the Italian Rice Research Institute in Vercelli, air-dried and stored at room temperature until the start of the experiment. Immediately prior to the establishment of microcosms, soil was sieved through a stainless steel screen (0.2 mm mesh) and 2.5 kg was added to opaque plastic pots. The pots were flooded with deionized water one week before planting. Fertilizers included urea (CH_4N_2O, 45 g l^{-1}) as nitrogen source, phosphorus ($Na_2HPO_4*2H_2O$, 17 g l^{-1}), potassium (KCl, 50 g l^{-1}) and magnesium ($MgSO_4*7H_2O$, 2 g l^{-1}). The phosphorus, potassium and magnesium solutions were added at a ratio of 10 ml kg^{-1} soil one day before planting, whereas 5 ml kg^{-1} of the urea was added twice, one day before planting and after 14 days of plant growth. Rice seeds (*Oryza sativa* var. Koral) were also obtained from the Rice Research Institute in Vercelli, Italy. The rice seeds were treated with the fungicide Aatiram and germinated at 25°C and 75% humidity in a greenhouse. Three germinated rice seedlings were planted each in a total of 20 pots. A further five pots were left unplanted. The pots were incubated in a greenhouse at 25°C and 75% humidity with a twelve hour light/dark cycle. Pots were watered daily to maintain approximately 3 cm water overlying the soil. Plant heights and tiller number were recorded weekly. Five planted pots were sacrificed after 34, 52, 62 and 90 days after planting and rhizosphere soil (planted pots), bulk soil (unplanted pots) and pore water were collected aseptically using sterilized equipment. Plants were extracted from the pots and shaken to remove large soil aggregates and adhering soil (bulk soil). The soil remained attached on the roots (rhizosphere soil) was sampled using a sterile spatula. Samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The water content of each soil was determined gravimetrically by drying subsamples at 60°C until they reached a constant weight.

2.3.2 Porewater analysis

100 g soil samples were centrifuged for 10 min at 20 000 *g* and 4°C in 50-ml centrifuge tubes. The supernatant was filter sterilized using 0.2 µm acetate-free filters (GE healthcare life science) and stored at -20°C until analysis. Organic acids were analyzed with

high performance liquid chromatography (HPLC, Krumböck and Conrad, 1991). Inorganic ions including chloride, nitrate, nitrite, phosphate and sulfate were detected using ion chromatography (Bak *et al*., 1991).

2.3.3 Nucleic acid extraction

Soil DNA was extracted using the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. DNA concentration and purity were determined spectrophotometrically (NanoDrop Technologies, USA). Only DNA samples with absorbance ratios of A260:A230 > 1.7 and A260:A280 > 1.8 were used for further analysis.

2.3.4 Real-time quantitative PCR

Quantitative PCR (qPCR) was used to quantify archaeal and bacterial 16S rRNA gene copies using primers Ba519f/Ba907r (Lane, 1991) and Ar364f/Ar934br (Burggraf *et al*., 1997; Großkopf *et al*., 1998), respectively. All qPCR reactions were pipetted on ice with minimal light exposure. Each reaction contained in a total volume of 25 µl: 4 mM $MgCl_2$, 0.01 µM fluorescein calibration dye (Bio-Rad), 0.625 U Jump Start™ Taq ReadyMix™, 0.60 µM of each primer, 1 µL template DNA. Standards containing known numbers of DNA copies of the target gene were serially diluted and used for construction of calibration curves in each reaction The qPCR reactions were performed on an iCycler thermocycler equipped with a MyiQ detection system (Bio-Rad, Munich, Germany). The following program was used for amplification of archaeal 16S rRNA gene copies: 94°C for 6 min, followed by 40 cycles of 94°C for 35 s, 66°C for 30 s and 72°C for 45 s and single step of final elongation of 86.5°C 10 s. A melting curve was performed from 75-95°C. The program for amplification of bacterial 16S rRNA gene copies was as follows: 94°C for 8 min, followed by 50 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 50 s. A melting curve was performed from 75-95°C. Data analysis was performed using BioRad IQ5 2.0 Standard Edition Optical System Software (Bio-Rad).

2.3.5 *16S rRNA amplicon pyrosequencing*

A total of 24 samples were chosen for amplicon pyrosequencing. Samples corresponded to three replicate microcosms chosen at random from each of the two

treatments (planted, unplanted) and the four sampling time points. The 16S rRNA genes of bacteria and archaea were targeted with primers F515 and R806 described by Bates *et al*. (2011). The forward primer was tagged with unique 6-base barcode. Sequencing of the PCR products was performed at the Max Planck Genome Centre in Cologne using a Roche 454 Genome Sequencer GS FLX+.

Sequences were grouped into OTUs (97% similarity) with UPARSE (Edgar 2013) according to the standard procedure recommended by the developer, with the exception that singletons were retained. Representative sequences of OTUs were classified in *mothur* version 31.2 (Schloss *et al*., 2009) using the silva taxonomy and the Wang (naïve Bayesian classifier) method. Relative abundances of OTUs between samples were analyzed using the *vegan* package version 2.0-10 (Oksanen *et al*., 2013) in R version 3.0.2 (R Development Core Team, 2011). Other statistical tests were performed using the R package *stats*. OTU abundances were standardized by a Hellinger transformation using the decostand function. Principle components analysis (PCoA) were performed based on Bray-Curtis dissimilarities. Principle components analysis (PCA) was performed using prcomp and the 50 OTUs contributing the largest absolute loadings in the first dimension were obtained from the rotation file. A heatmap corresponding to the relative abundance of these 50 OTUs was prepared using the gplots package (version 2.16.0). The samples were clustered with hclust using the 'Ward' method based on Manhattan distances calculated with vegdist. Phylogenetic trees were calculated by aligning OTU sequences with sina (Pruesse *et al*., 2012) and added to the SILVA119 NR reference tree (Quast *et al*., 2013) by parsimony in ARB (Ludwig *et al*., 2004). A randomly subsampled dataset corresponding to 3707 reads per sample was obtained using the "sub.sample" script in *mothur*. This subsampled data was used for metastats analysis (White *et al*., 2009), which was performed with *CloVR Metagenomics 1.0* software platform (Angiuoli *et al*., 2011). Only OTUs displaying P-values < 0.01 were retained.

2.3.6 Terminal restriction fragment length polymorphism (T-RFLP)

Archaeal 16S rRNA genes were amplified by PCR using the primers Ar109f (Grosskopf *et al*., 1998) and Ar912rt (Lueders and Friedrich, 2002) with the FAM (6-carboxyfluorescein) on the reverse primer. All primers were obtained from (Sigma-Aldrich,

Taufkirchen, Germany). The PCR reactions were performed in a total volume of 50 µl containing 60 µM dNTPs (Fermentas, St. Leon-Rot, Germany), 1 x PCR-Buffer S (PeqLab, Erlangen, Germany), 0.5 µM of each primer, 0.2 mg ml^{-1} Bovine Serum Albumin (Roche, Grenzach, Germany), 1 U PeqLab Taq DNA polymerase (PeqLab) and 1 µl of DNA template (~ 20 ng/ µl). PCR amplification was performed using a GenAmp 9700 Thermocycler (Applied Biosystems, Carlsbad CA, USA) as follows: 94°C for 2 min, followed by 30-35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 60 s and a final elongation of 72°C for 7 min. The PCR products were purified using MinElute Purification Kit (Qiagen, Hilden, Germany). Archaeal genes were digested with *TaqI* (Promega) for 3 h at 37°C. Digested DNA was purified using SigmaSpin™ post-reaction clean-up columns (Sigma-Aldrich). For T-RFLP, 2 to 4 µl digested DNA were mixed with 11 µl deionized HiDi formamide (Applera Deutschland GmbH, Darmstadt, Germany) and 0.2 µl of the internal DNA standard (X-Rhodamine MapMarker® 1000, BioVentures, Murfreesboro, Tennessee, USA). After DNA denaturation at 95°C for 3 min using a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany), capillary size separation was performed with an ABI PRISM 3130 Genetic Analyzer (Applera Deutschland GmbH). Electropherograms were analyzed using Genescan 4.0 software (Applied Biosystems, Carlsbad CA, USA) and the T-RFLP profiles were standardized as described in Dunbar *et al.* (2001).

2.4 Results

2.4.1 Characterization of microcosms

The determination of the growth stage was conducted by monitoring rice plant height and tiller number (data not shown). Four sampling time points were selected corresponding to early vegetative (day 34), late vegetative (day 52), reproductive (day 62) and maturity (day 90) of rice plants. Soil pore water analyses indicated that lactate, formate, acetate, chloride and propionate concentrations were higher in the planted pots, but these differences were only statistically significant for chloride and propionate (Supplement Table 2.1). The concentrations of malate, nitrate and sulfate were similar between planted and unplanted pots.

2.4.2 Bacterial and archaeal 16S rRNA abundance

The influence of the rice plant on the microbial community size was determined by qPCR assays targeting the bacterial and archaeal 16S rRNA gene. Both bacterial and archaeal 16S rRNA abundances were about two-fold higher in rhizosphere (planted pots) than in soil from unplanted pots (Table 2.1). The 16S rRNA gene copy numbers of Bacteria were about 20 times more numerous than those of Archaea.

Table 2.1 Copy numbers of bacterial and archaeal 16S rRNA genes in planted and unplanted soil during the four sampled time points. Flooding period 1= 34 days; 2= 52 days; 3= 62 days ; 4= 90 days. Same letters indicate that microbial communities are not significantly different (ANOVA, P< 0.05). n=5, gdw= gram dry weight of soil.

Flooding period	Domain	Sample type	Copy number/gdw (± std.error)
1	Bacteria	Planted	$6.6x10^{9\ a}$ ($\pm 1.5x10^{9}$)
		Unplanted	$2.7x10^{9\ a}$ ($\pm 8.7x10^{8}$)
	Archaea	Planted	$3.2x10^{8\ a}$ ($\pm 1.0x10^{8}$)
		Unplanted	$1.1x10^{8\ a}$ ($\pm 1.8x10^{7}$)
2	Bacteria	Planted	$5.8x10^{9\ a}$ ($\pm 1.8x10^{9}$)
		Unplanted	$2.0x10^{9\ a}$ ($\pm 5.0x10^{9}$)
	Archaea	Planted	$2.5x10^{8\ a}$ ($\pm 5.7x10^{7}$)
		Unplanted	$8.3x10^{7\ b}$ ($\pm 2.1x10^{7}$)
3	Bacteria	Planted	$3.7x10^{9\ a}$ ($\pm 7.2x10^{8}$)
		Unplanted	$2.5x10^{9\ a}$ ($\pm 9.1x10^{8}$)
	Archaea	Planted	$1.7x10^{8\ a}$ ($\pm 2.3x10^{7}$)
		Unplanted	$8.2x10^{7\ a}$ ($\pm 2.0x10^{7}$)
4	Bacteria	Planted	$3.3x10^{9\ a}$ ($\pm 1.1x10^{9}$)
		Unplanted	$1.7x10^{9\ a}$ ($\pm .3.6x10^{8}$)
	Archaea	Planted	$1.7x10^{8\ a}$ ($\pm 2.4x10^{7}$)
		Unplanted	$1.1x10^{8\ a}$ ($\pm 1.5x10^{7}$)
All	Bacteria	Planted	$4.8x10^{9\ a}$ ($\pm 1.4x10^{9}$)
		Unplanted	$2.4x10^{9\ b}$ ($\pm 6.9x10^{8}$)
	Archaea	Planted	$2.2x10^{8\ a}$ ($\pm 5.9x10^{7}$)
		Unplanted	$9.7x10^{7\ b}$ ($\pm 1.9x10^{7}$)

2.4.3 Bacterial and archaeal diversity

Pyrosequencing of the bacterial 16S rRNA gene was performed to identify the phylogenetic groups influenced by the flooding period or the presence of the rice plant. A total of 175501 sequences were obtained after preprocessing and chimera removal. The sequences grouped into 8685 OTUs at 97% similarity.

Sequences were classified and summarized by bacterial phylum, which did not show any significant difference between planted and unplanted soil samples (Figure 2.1) indicating the overall structure of the community at low phylogenetic resolution did not change between these environments. The microbial communities did separate by planted and unplanted based on the principle coordinates analysis (PCoA) of OTUs (Figure 2.2),

indicating that there was a change in the community structure at higher (97% identity) phylogenetic resolution. Despite this clear separation, the first axis only explained 8.6% of the variance in the data. There was no apparent clustering of rhizosphere samples based on plant growth stage or the corresponding sampling time in unplanted soil microcosms. Metastats analysis was used to quantify the extent to which OTUs differed between planted and unplanted samples. A total of 64 OTUs were identified as having significantly higher relative abundance in unplanted pots (Table 2.2). This corresponded to ~1% of OTUs and 6.4% of reads. Similarly, 87 OTUs had higher relative abundance in the rhizosphere samples, corresponding to an average of 9.9% of reads in those samples (Table 2.3). These analyses indicated that there were differences between the soil communities in planted and unplanted microcosms, but in general these were few and characterized by subtle shifts in relative abundance of OTUs.

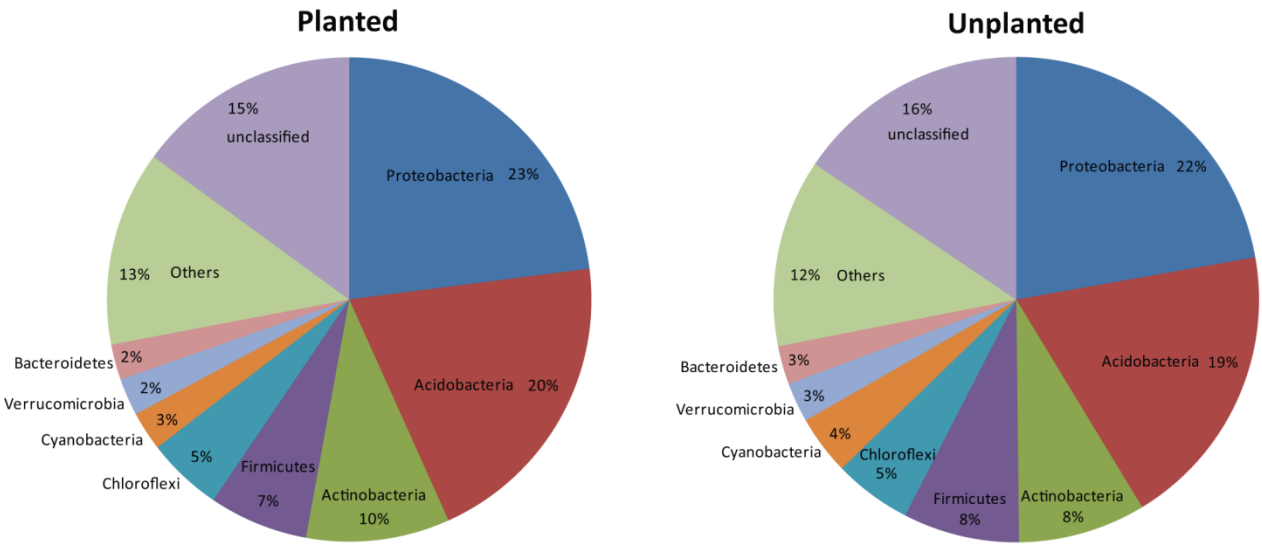


Figure 2.1. Phylum level comparison of bacteria abundance in planted and unplanted pots based on 454 pyrosequencing analysis of 16S rRNA genes. All phyla contributing less than 2% of sequences were summarized as others.

Table 2.2 Taxonomy of OTUs with significantly higher relative abundance (metastats, P<0.01) in unplanted than planted soil. The number of OTUs corresponding to each taxon is indicated and their average cumulative relative abundance within each taxon in planted and unplanted soil are indicated.

		OTUs	*Unplanted (%)*	*Planted (%)*
Acidobacteria	Gp1	3	0.26	0.12
	Gp16	2	0.08	0.02
	Gp18	1	0.03	0.00
	Gp3	2	0.09	0.03
	Gp7	1	0.05	0.02
Actinobacteria	Actinomycetales	1	0.07	0.01
	Solirubrobacterales	2	0.06	0.02
	Acidimicrobiales	1	0.02	0.00
	unclassified	1	0.03	0.01
Armatimonadetes	Gp2	2	0.04	0.00
	Chthonomonadaceae	1	0.12	0.03
Bacteroidetes	Chitinophagaceae	2	0.13	0.03
Chlorobi	Ignavibacterium	1	0.04	0.01
Chloroflexi	Anaerolineaceae	1	0.07	0.02
Cyanobacteria	Order *	1	0.04	0.00
Firmicutes	Paenibacillaceae 2	1	0.24	0.14
	Tissierella	1	0.02	0.00
	Clostridium sensu stricto	1	0.03	0.00
Gemmatimonadetes	Gemmatimonas	7	1.01	0.45
Alphaproteobacteria	Rhodospirillales	2	0.08	0.01
	Porphyrobacter	1	0.04	0.01
	unclassified	2	1.00	0.73
Betaproteobacteria	Nitrosospira	1	0.09	0.05
	Burkholderiales	1	0.02	0.00
	unclassified	5	0.91	0.48
Deltaproteobacteria	Anaeromyxobacter	1	0.42	0.23
	Myxococcales	4	0.20	0.05
Gammaproteobacteria	Steroidobacter	1	0.03	0.00
	Dokdonella	1	0.14	0.08
	unclassified	3	0.19	0.07
Unclass. Proteobacteria		1	0.22	0.12
Unclassified		7	0.32	0.09
Verrucomicrobia	Spartobacteria genera*	1	0.25	0.13
	Subdivision3 family*	1	0.05	0.00
	Sum	64	6.39	2.97

*=incertae sedis

A heatmap was constructed to depict the relative abundance of OTUs that best represent the dissimilarity between the microbial communities in planted and unplanted microcosms. A total of 50 OTUs were selected, of which 34 could be classified to family level or lower and included in the heatmap (Figure 2.3). Most of the OTUs were also identified as significantly different ($P < 0.01$) in the metastats analysis (results not shown). These 50 OTUs alone were able to distinguish the planted and unplanted soil microbial

communities, as seen by the clustering of the samples. The rhizosphere soil contained a significantly higher abundance of OTUs corresponding to the rice mitochondria and plastid 16S rRNA sequences, which likely arose from root cells sloughed-off into the rhizosphere. The clustering of unplanted soil communities suggested a separation into early and late sampling time, whereas no temporal pattern was apparent for the rhizosphere samples. Among the OTUs that appeared to shift in relative abundance from early to late in the unplanted soil included a *Nitrosospira* and a *Gemmatimonas* taxon. *Cyanobacteria* in particular tended to appear sporadically among samples rather than being evenly enriched between replicates.

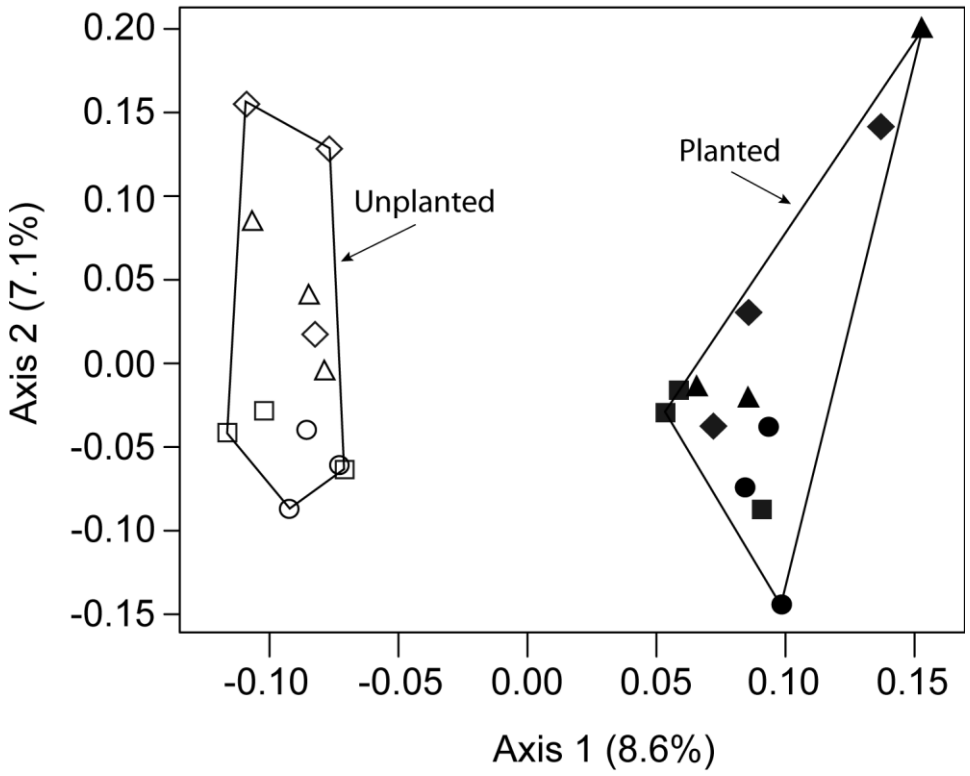


Figure 2.2 Principal coordinate analysis (PCoA) ordination based on relative abundance of 16S rRNA gene OTU abundances. Symbols represent sampling times according to plant age: square (stage 1, 34 days), triangles (stage 2, 52 days), circles (stage 3, 62 days) and diamonds (stage 4, 90 days). Filled samples correspond to unplanted and open symbols to planted soil.

Table 2.3 Taxonomy of OTUs with significantly higher relative abundance (metastats, P<0.01) in planted than unplanted soil. The number of OTUs corresponding to each taxon is indicated with the average cumulative relative abundance of each taxon in planted and unplanted soil indicated.

		OTUs	Planted (%)	Unplanted (%)
Euryarchaeota	Methanoregula	1	0.02	0.00
Acidobacteria	Gp16	2	1.60	1.16
	Gp23	1	0.02	0.00
	Gp4	1	0.46	0.27
	Gp7	3	1.05	0.66
Actinobacteria	Actinobacteria	1	0.09	0.04
Bacteroidetes	Unclassified	5	0.35	0.07
Chloroflexi	Anaerolineaceae	1	0.02	0.00
Cyanobacteria	Streptophyta	1	0.13	0.01
	Chlorophyta	1	0.03	0.00
	GpV	1	0.03	0.00
	Cyanobacteria order*	1	0.02	0.00
Firmicutes	Thermoactinomyces	1	0.10	0.04
	Clostridium sensu stricto	2	0.22	0.12
	Clostridium	1	0.07	0.01
	Clostridiales	1	0.21	0.07
	Clostridiaceae	1	0.07	0.00
	Veillonellaceae	2	0.09	0.02
	Clostridia	2	0.08	0.02
Gemmatimonadetes	Gemmatimonas	1	0.07	0.01
Alphaproteobacteria	Azospirillum	1	0.02	0.00
	Magnetospirillum	3	0.09	0.02
	Dongia	1	0.02	0.00
	Mitochondria genus*	1	0.06	0.01
	Rhizobiales	1	0.08	0.02
Betaproteobacteria	Comamonadaceae	1	0.19	0.02
	Burkholderiales	1	0.50	0.07
	Rhodocyclaceae	2	0.16	0.01
	Unclassified	1	0.09	0.02
Deltaproteobacteria	Geobacter	6	0.91	0.31
	Cystobacteraceae	1	0.11	0.02
	Anaeromyxobacter	4	0.68	0.32
Gammaproteobacteria	Methylomonas	1	0.02	0.00
	Xanthomonadaceae	1	0.02	0.00
	Unclassified	1	0.09	0.02
Unclassified		26	1.76	0.47
Verrucomicrobia	Opitutaceae	2	0.20	0.00
	Opitutus	2	0.16	0.02
	Spartobacteria genera*	1	0.02	0.00
	Sum	87	9.91	3.83

*=incertae sedis

Among these 50 OTUs, we then selected the ten with the highest contribution to the original PCA ordination and repeated the PCA separately using their relative abundance in unplanted (Figure 2.4A) or planted samples (Figure 2.4B). The ordination of the unplanted samples appeared to separate by early and late time points along the second axis of the ordination. In the ordination using the rhizosphere samples the replicates from each time point did not overlap, suggesting that their relative abundance shifted by plant growth stage. These OTUs included several that could not be classified below the level of order using the naïve Bayesian classifier, and therefore we added them to a phylogenetic tree to indicate their closest relatives (Figure 2.4c). All the OTUs belonged to *Proteobacteria*, with the exception of OTU_73 that could not be clearly assigned to any bacterial phylum.

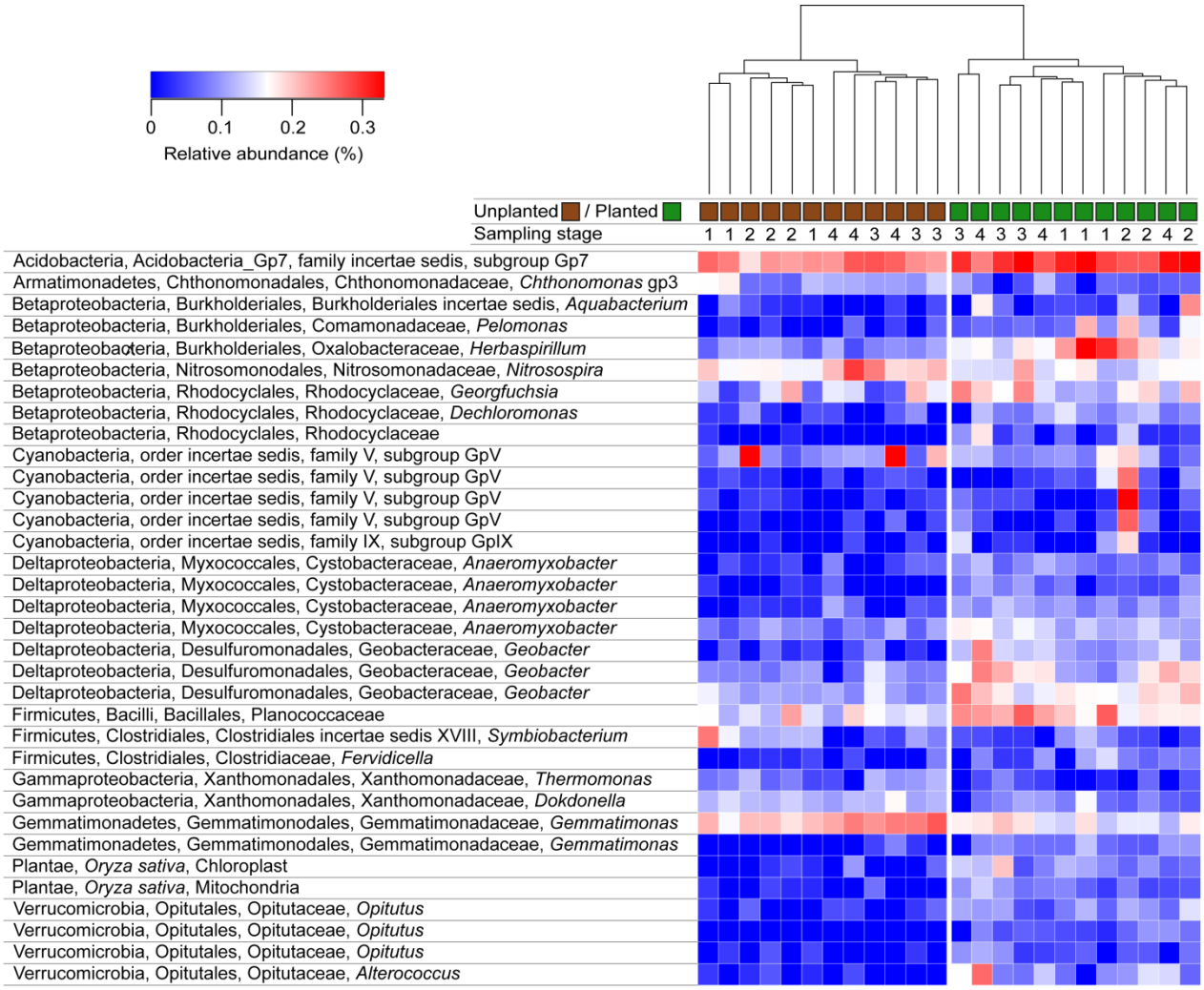


Figure 2.3 Heatmap depicting the relative abundance of OTUs with greatest dissimilarity between planted and unplanted microcosms. Samples were clustered based on Manhattan distances. OTUs were classified and only those that could be classified to order or lower were included. The sampling times (1-4) correspond to 34, 52, 62 and 90 days after planting.

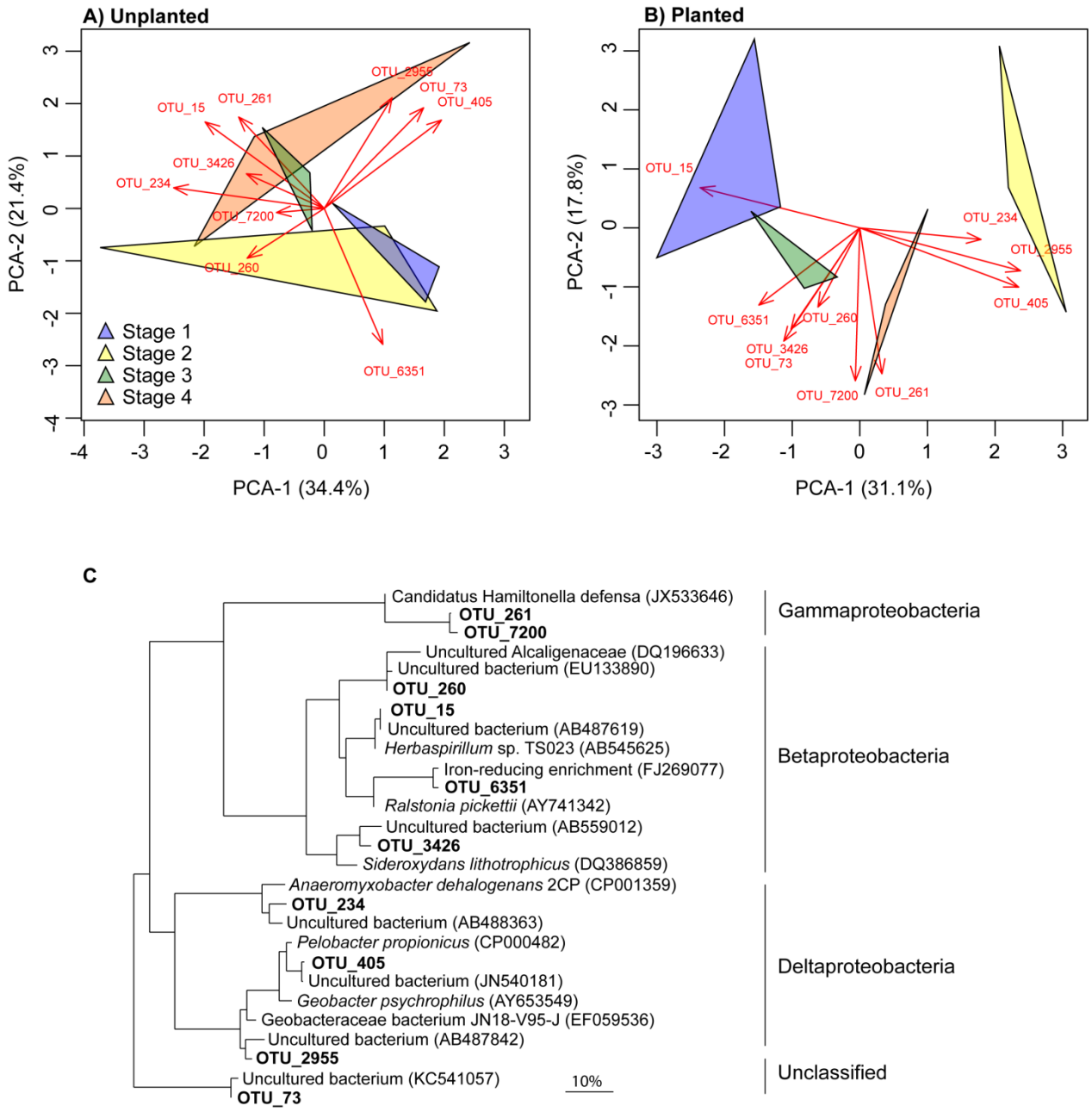


Figure 2.4 Analysis of ten top OTUs explaining difference between planted and unplanted microbial communities. PCoA of the ten OTUs in the unplanted (**A**) and planted (**B**) samples. The corners of the triangles correspond to the positions of the triplicates. Phylogenetic tree (**C**) for the ten OTUs showing representatives of their closest relatives in the Silva 16S rRNA database.

Although the metastats analysis and the PCA/heatmap provided hints as to which bacterial phyla were differentially abundant between planted and unplanted soil microcosms, we wanted to determine if this pattern could be seen for individual bacterial phyla alone. To determine this, OTUs from bacterial phyla were selected and analyzed individually by PCoA. The dissimilarity between planted and unplanted soil was seen most notably for *Acidobacteria, Bacteroidetes, Gemmatimonadetes, Proteobacteria* and *Verrucomicrobia* (Figure 2.5). The distribution of samples from several phyla, such as *Actinobacteria, Armatimonadetes, Cyanobacteria* and *Planctomycetes* overlapped in the ordination and the BRC phylum showed no apparent separation of planted and unplanted communities along either the first or second axis. Archaea also showed no separation based on planted and unplanted microcosms (Figure 2.6a). Since Archaea represented only 1.2% of reads, one possibility was that the relatively poor coverage hampered the statistical power to resolve patterns between planted and unplanted samples. Therefore, we repeated the analysis using Archaea-specific 16S rRNA primers and T-RFLP. Again, no difference between planted and unplanted samples was seen in the in the PCoA ordination (Figure 2.6b), indicating that indeed the distribution of Archaea did not differ between rhizosphere and unplanted soil, despite an increase in their absolute abundance (Table 2.1).

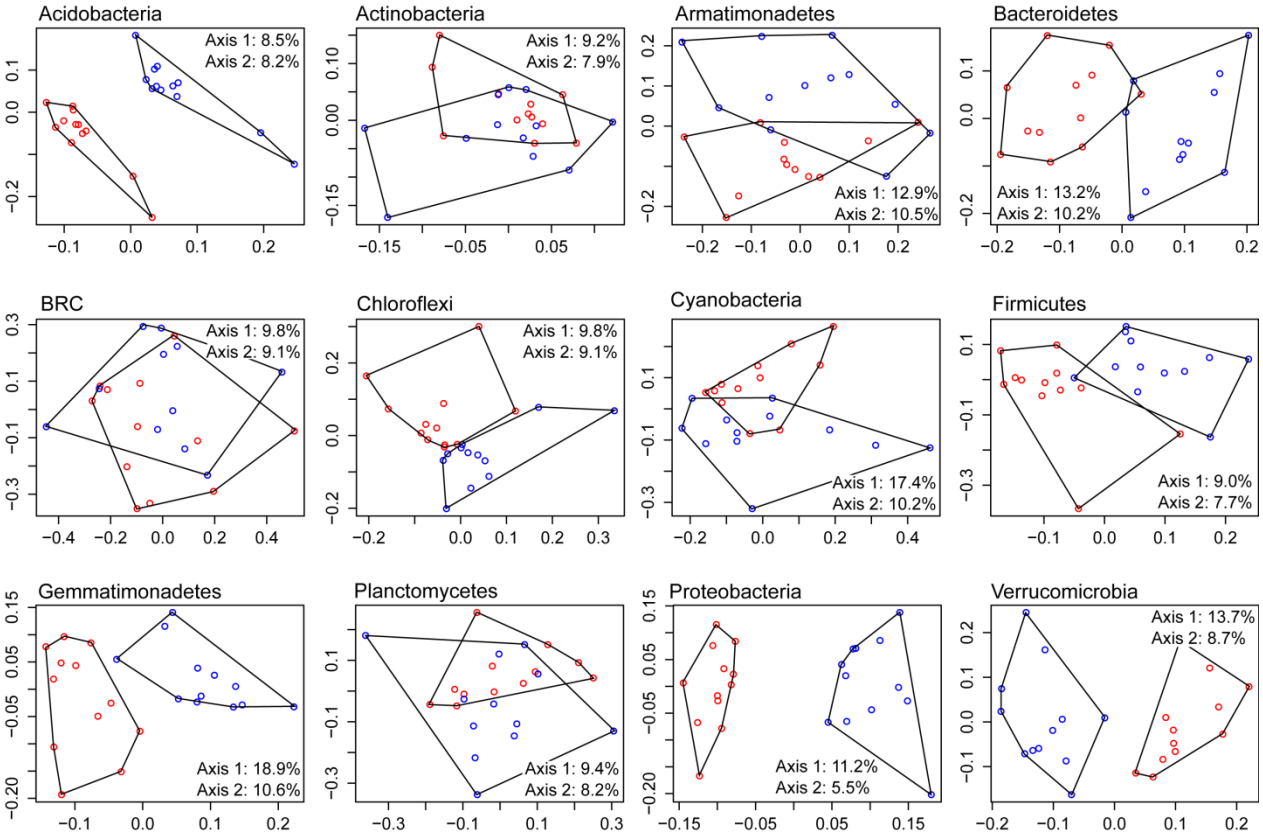


Figure 2.5 PCoA analysis of 16S rRNA gene OTUs assigned to major bacterial phyla detected in Vercelli rice field soil. Unplanted samples are depicted with red symbols and planted samples in blue.

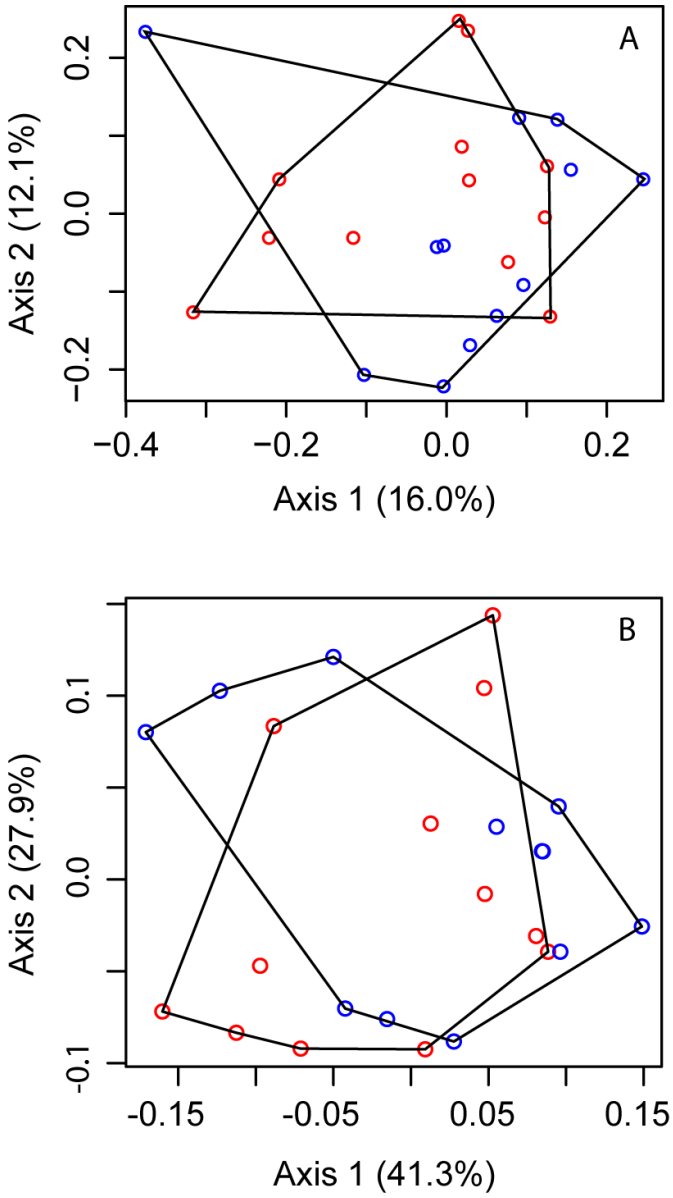


Figure 2.6 PCoA analysis of archaeal OTUs. (**A**) Pyrosequencing data using universal 16S rRNA primers, and (**B**) T-RFLP analysis using Archaea-specific primers. Unplanted samples are depicted with red symbols and planted samples in blue.

2.5 Discussion

The microbial community in the rhizosphere is known to be influenced and shaped by the plant, but the exact nature of the change and the extent to which this occurs in a single growing season of rice was not known. Therefore we examined the size and composition of the community in the rhizosphere at different plant growth stages. We compared this to unplanted rice soil microcosms to control for changes in the community resulting from soil flooding and fertilization, such as the sequential enrichment of microbes able to exploit different electron acceptors (Shrestha *et al*., 2007). The key finding was that the greatest difference in microbial community was between planted (i.e. rhizosphere) and unplanted soil (Figure 2.2), with only minor changes occurring over time. This indicates that the plant is the major driver of microbial community composition in rice field soil. In addition, the differences between the planted and unplanted microbial communities were relatively subtle.

The rhizosphere is generally known as a compartment in the soil influenced by the plant and where organic matter is introduced via rhizodeposition and sloughed-off cells. Rhizodeposition by rice plants has been described (e.g. Aulakh *et al*., 2001; Wu *et al*., 2008) and shown to enhance microbial activity in the rhizosphere compared with bulk soil (Butler *et al*. 2003). Oxygen is also released from rice roots (e.g. Armstrong, 1979; Frenzel *et al*., 1992) and serves as an electron acceptor for aerobic microorganisms. The volume and composition of organic molecules released from roots changes somewhat with rice plant growth stage (Aulakh *et al*., 2001), which in theory could also cause temporal shifts in the rhizosphere microbial community. Our data (Supplement Table 2.1) and other studies show that the combined influence of the rice plant and the microorganisms in the rhizosphere act to modify the chemical composition of the soil environment. We show that the abundance of Archaea and Bacteria were two-fold higher in the rice rhizosphere than the bulk soil of the unplanted pots (Table 2.1).

Although there was a doubling of the bacterial community in the rhizosphere as determined by qPCR targeting bacteria 16S rRNA genes, there was no significant difference in the relative abundance of bacterial at the phylum level (Figure 2.1). This indicates that this increase in abundance occurred evenly, otherwise the relative abundance of the stimulated phyla would have increased and unresponsive phyla would have decreased relative to the

unplanted control. Without targeted qPCR assays for individual phyla we lack the statistical power to prove that all the bacterial phyla were stimulated in the rhizosphere, but we can conclude that the stimulation of microbial growth in the rhizosphere was not limited to a small subset of phyla. To explore this further we focused on a comparison of OTUs (97% sequence identity) within individual phyla to determine if there was a differential response by phylum members. The within-phylum community structure was clearly affected by the rice plant in several cases, but most notably *Acidobacteria, Gemmatimonadetes, Planctomycetes, Proteobacteria* and *Verrucomicrobia* (Figure 2.5). The clear separation of planted and unplanted communities for these phyla along the first axis of the ordinations indicates differential effects of the rice plant on the phylum members, and that the rice plant was the primary factor affecting the relative abundance of OTUs.

In the case of Archaea, their abundance was on average more than two-fold greater in rhizosphere than unplanted soil (Table 2.1), but the archaeal community composition was apparently not differentially shaped by these locations (Figure 2.6). An increase in the absolute abundance of a group without a change in the relative abundance of the individual OTUs would indicate an equal stimulation of all taxa, which appeared to be the case of Archaea in our experimental system. Studies have shown that archaeal communities are relatively stable once established and are resistant to environmental perturbation, such as drainage (**Chapter 3**, Breidenbach and Conrad, 2015; Krüger *et al*., 2005; Scavino *et al*., 2013; Watanabe *et al*., 2006), or oxygenation in the case of strict anaerobes (Yuan *et al*., 2011). The lower abundance of Archaea in the unplanted soil compared with rhizosphere is a reflection of energy or carbon limitation in the absence of rice plants. Our current hypothesis is that the relative abundance of Archaea in Vercelli rice field soil is shaped by their growth in the rhizosphere, and that archaeal OTUs respond proportionally to carbon or energy limitation.

The overall archaeal and bacterial communities in the planted and unplanted pots were mainly similar possibly indicating a pronounced influence of the soil type and conditions. Rice field soil is mainly flooded during rice cultivation resulting in anoxic soil compartments which are colonized by anaerobic microbes. The soil we used in the present study has been used for rice cultivation for more than 100 years

(http://sito.entecra.it/portale/cra_dati_istituto.php?id=226&lingua=EN). It is likely that the microbial communities inhabiting the soil are highly adapted to the anoxic conditions and are shaped by rice plant nutrient inputs since rice is cultivated as a monoculture in these fields. The soil is typically drained after harvest and therefore is accustomed to a cycle of dessication followed by flooding. Similarly, the soil was dried in the laboratory prior to the experiment and both planted and unplanted microcosms were fertilized, as is also common practice for the cultivation of rice in Vercelli rice fields. As water regime and fertilization were equal in both planted and unplanted soil, the different patterns observed result either directly or indirectly from the rice plant.

In both planted and unplanted soil we were not able to detect dramatic temporal changes in the microbial community composition. This may indicate that this community is highly adapted to nutrients available and that for example species involved in the anaerobic breakdown of organic matter are specialized in the Vercelli rice field soil. The sequential enrichment of microbes as a consequence of the availability of different electron acceptors (Shrestha *f*., 2007) was not clearly observed in the present study. However, these processes occur shortly after flooding when electron acceptors such as oxygen and nitrate are rapidly depleted (Conrad and Frenzel, 2002). Therefore, it is likely that these sequential dynamics occurred before our first sampling on day 34. An analysis of the ten OTUs with the greatest dissimilarity between planted and unplanted microcosms did suggest a temporal change in the rhizosphere according to plant age (Figure 2.4b), supporting the hypothesis that changes in rhizodeposition during rice plant growth may impact microbes in the rhizosphere. Also, the unplanted soil revealed slight differences in relative abundances of OTUs between early and late sampling time points (e.g. Figure 2.3, Figure 2.4).

Despite the overall bacterial community was similar in planted and unplanted soil we found specific bacterial groups differing. In the planted soil we found bacterial lineages which likely were influenced by the oxygen supply of the rice plant such as *Rhizobiales*, which are known aerobes (Yanagi and Yamasto, 1993) and have also been identified a capable of consuming rice plant-derived carbon (Hernández *et al*., 2015). Furthermore, the anaerobic iron reducers *Geobacter* and *Anaeromyxobacter* were found enriched in the planted soil. Oxidants such as ferric iron are rapidly reduced in rice field soils (Conrad and

Frenzel, 2002), but may be regenerated when plants release O_2 from their roots. The second group of bacteria found enriched in the planted soil may be support the rice plant growth by providing nitrogen and sulfur compounds such as *Herbaspirillum*, *Burkholderia* and *Comomonas*. *Herbaspirillum* and *Burkholderia* spp. are both capable of nitrogen fixation and reported to increase biological nitrogen fixation in rice plants (Baldani *et al*., 2000). Also, *Burkholderia* were identified to actively assimilate rice plant derived carbon (Lu *et al*., 2006). *Comomonas* were recently identified enriched on rice roots (Hernández *et al*., 2015) and might have a role in supplying plants with sulfur via desulfonation reactions (Inceoglu *et al*., 2010). Finally, *Opitutus* and *Clostridia* were found to be enriched in the rhizosphere compared with unplanted soil, and were both identified consumers of plant-derived carbon (Hernández *et al*., 2015). Both these organisms are strict anaerobes with fermentative metabolism (Andreesen *et al*., 1973, Chin *et al*., 2001).

The bacterial lineages found in higher abundance in the unplanted soil can be divided into two groups: (I) anaerobes degrading complex carbon compounds adapted to low substrate conditions and (II) aerobic organisms. The first group was composed of *Actinobacteria* which were suggested to be involved in decomposition of less-degradable compounds (Rui *et al*., 2001) and recently identified to be involved in hemicellulose breakdown in rice field soil (Wegner and Liesack., 2015). The second group comprises *Gemmatimonadetes*, *Nitrosospira* and *Spartobacteria*. Recently, *Spartobacteria* were reported to using plant carbon colonizing the rice roots (Hernández *et al*., 2015). The aerobic *Chthoniobacter flavus* is one of the only characterized isolates of *Spartobacteria* and was shown to grow on saccharides including starch and cellulose (Sangwan *et al*., 2004). *Nitrosospira* spp. are represented by aerobic ammonia-oxidizing bacteria and have been isolated from the rhizoplane of rice (Aleem *et al*., 1965; Tomiyama *et al*., 2001). The higher relative abundance of a *Nitrosospira* OTU in the unplanted soil compared to the rhizosphere might be a reflection of less competition for ammonia in the absence of rice plants. Lastly, there are only two isolates belonging to the *Gemmatimonadetes* and both are aerobes capable of polyphosphate accumulation (Zhang *et al*., 2003; DeBruyn *et al*., 2013). It is possible that these OTUs were enriched during soil storage prior to start of the experiment, after which their relative abundance decreased in the rhizosphere as the absolute abundance of the total bacterial community increased in this zone.

The results of this study indicate that the rice plant is the major driver of microbial community composition in rice field soil and that this community is highly adapted to the specific conditions of rice plant growth. Future studies could explore this by cultivating other crops in Vercelli rice field soil to determine if this results in a relatively large shift in the rhizosphere microbial community. Furthermore, it would be intriguing to expose Vercelli soil to repeated cycles of flooding without rice plants to determine if the historical influence of rice cultivation begins to dissipate and a more pronounced shift in the microbial community structure is observed.

2.6 Supplemental material

Supplement Table 2.1 Concentrations of organic acids and inorganic ions detected in the porewater of planted and unplanted pots. Values indicate the average and standard deviation in millimolar concentrations. Flooding period 1= 34 days; 2= 52 days; 3= 62 days ; 4= 90 days. Values with the same letters indicate that organic acid and inorganic ion concentrations are not significantly different (P< 0.05) by ANOVA. ND indicates not detected.

Flooding period	Malate	Lactate	Formate	Acetate	Propionate	Chloride	Nitrate	Sulfate
Planted								
1	70.23 ± 23.05[a]	0.98 ± 1.01[a]	0.13 ± 0.14[a]	0.44 ± 0.42[a]	0.06 ± 0.09	12.70 ± 4.08 [a]	0.02 ± 0.02[a]	0.03 ± 0.03[a]
2	69.18 ± 25.17[a]	1.41 ± 1.39[a]	0.10 ± 0.10[a]	0.50 ± 0.56[a]	1.17 ± 1.69	30.95 ± 2.40 [b, c]	0.01 ± 0.01[a]	0.02 ± 0.02[a]
3	103.51 ± 11.45[a]	1.16 ± 0.40[a]	0.31 ± 0.14[a]	0.48 ± 0.41[a]	0.38 ± 0.20	29.06 ± 5.98 [b ,c]	0.05 ± 0.05[a]	0.02 ± 0.01[a]
4	121.92 ± 19.63[a]	1.26 ± 0.53[a]	0.40 ± 0.09[a]	0.55 ± 0.41[a]	0.96 ± 0.35	34.91 ± 2.08 [b]	Nd	0.03 ± 0.02[a]
Unplanted								
1	85.96 ± 9.43[a]	0.17 ± 0.18[a]	0.03 ± 0.05[a]	0.02 ± 0.04[a]	Nd	12.05 ± 1.07 [a]	0.13 ± 0.20[a]	0.02 ±0.02[a]
2	101.31 ± 8.37[a]	0.01 ± 0.02[a]	0.09 ± 0.02[a]	0.01 ± 0.01[a]	Nd	14.49 ± 1.64 [a]	0.05 ± 0.05[a]	0.02 ±0.02[a]
3	119.89 ± 58.70[a]	0.03 ± 0.07[a]	0.05 ± 0.03[a]	Nd	0.03 ± 0.00	16.48± 3.36 [a]	0.14 ± 0.15[a]	0.03 ± 0.02[a]
4	148.13 ± 88.17[a]	0.04 ± 0.07[a]	0.04 ± 0.03[a]	0.01 ± 0.01[a]	Nd	19.10 ± 4.52 [a, c]	0.23 ± 0.30[a]	0.05 ±0.03[a]

2.7 Acknowledgements

We thank Dr Elisabetta Lupotto (CRA-RIS, Vercelli, Italy) for providing rice seeds and soil. B. B. was financially supported the German Research Foundation (DFG) for funding (FOR 1701, ‘‘Introducing Non-Flooded Crops in Rice-Dominated Landscapes: Impacts on Carbon, Nitrogen and Water Cycles [ICON]”. J.P. was financially supported by the Research Center for Synthetic Microbiology (‘Synmikro’) of the Landes-Offensive zur Entwicklung wissenschaftlich-ökonomischer Exzellenz (LOEWE).

2.8 References

Aleem, M. I., Hoch, G. E., and Varner, J. E. (1965). Water as the source of oxidant and reductant in bacterial chemosynthesis. *Proc. Natl. Acad. Sci. USA* 54, 869-873.

Andreesen, J. R., Schaupp, A., Neurauter, C., Brown, A., and Ljungdahl, L. G. (1973). Fermentation of glucose, fructose, and xylose by *Clostridium thermoaceticum*: effect of metals on growth yield, enzymes, and the synthesis of acetate from CO_2. *J. Bacteriol.* 114, 743-751.

Angiuoli, S. V., Matalka, M., Gussman, A., Galens, K., Vangala, M., Riley, D. R., *et al*. (2011). CloVR: a virtual machine for automated and portable sequence analysis from the desktop using cloud computing. *BMC Bioinformatics* 12, 356. doi:10.1186/1471-2105-12-356.

Armstrong, W. (1979) Aeration in higher plants. *Adv. Bot. Res.* 7, 226-332.

Aulakh, M. S., Wassmann, R., Bueno, C., Kreuzwieser, J., and Rennenberg, H. (2001). Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biol*. 3, 139-148. doi: 10.1055/s-2001-12905

Badri, D.V., and Vivanco, J. M., (2009). Regulation and function of root exudates. *Plant Cell Environ*. 32, 666-681.

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., and Vivanco, J. M., (2004). How plants communicate using the underground information superhighway. *Trends Plant Sci*. 9, 26-32.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M., (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev.Plant Biol*. 57, 233-266.

Bak, F., Scheff, G., and Jansen, K. H. (1991). A rapid and sensitive ion chromatographic technique for the determination of sulfate and sulfate reduction rates in freshwater lake sediments *FEMS Microbiol. Lett.* 81, 23-30.

Baldani, V. D., Baldani, J. I., and Döbereiner, J. (2000). Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. *Biol. Fertil. Soils* 30, 485-491.

Bates, S. T., Cropsey, G. W., Caporaso, J. G., Knight, R., and Fierer, N. (2011). Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol*. 77, 1309-1314. doi: 10.1128/AEM.02257-10.

Breidenbach, B., and Conrad, R. (2015). Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Front. Microbiol*. 5, 752. doi: 10.3389/fmicb.2014.00752.

Burggraf, S., Huber, H., and Stetter, K. O. (1997). Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *Int. J. Syst. Evol. Microbiol*. 47, 657-660.

Butler, J. L., Williams, M. A., Bottomley, P. J., and Myrold, D. D. (2003). Microbial community dynamics associated with rhizosphere carbon flow. *Appl. Environ. Microbiol*. 69, 6793-6800.

Chin, K. J., Liesack, W., and Janssen, P. H. (2001). *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division '*Verrucomicrobia*' isolated from rice paddy soil. *Int. J. Syst. Evol. Micr*. 51, 1965-1968.

Conrad, R., and Frenzel, P. (2002). "Flooded soils". in: *Encyclopedia of Environmental Microbiology*, ed. G. Britton, John Wiley & Sons, New York, USA, 1316-1333. doi:10.1002/0471263397.env034.

DeBruyn, J. M., Fawaz, M. N., Peacock, A. D., Dunlap, J. R., Nixon, L. T., Cooper, K. E., and Radosevich, M. (2013). *Gemmatirosa kalamazoonesis* gen. nov., sp. nov., a member of the rarely-cultivated bacterial phylum *Gemmatimonadetes*. *J. Gen. Appl. Microbiol*. 59, 305-312.

Dunbar, J., Ticknor, L. O., and Kuske, C. R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol*. 67, 190-197.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996-998.

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., *et al*. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci.* 112, E911-E920.

Fernandez Scavino, A., Ji, Y., Pump, J., Klose, M., Claus, P., and Conrad, R. (2013). Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated rice fields. *Environ. Microbiol*. 15, 2588-2602.

Frenzel, P., Rothfuss, F., and Conrad, R. (1992). Oxygen profiles and methane turnover in a flooded rice microcosm. *Biol. Fertil. Soils* 14, 84-89.

Großkopf, R., Janssen, P. H., and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64, 960-969.

Haichar, Z. F., Santaella, C., Heulin, T., and Achouak, W. (2014). Root exudates mediated interactions belowground. *Soil. Biol. Biochem.* 77, 69-80.

Hawes, M. C., Gunawardena, U., Miyasaka, S., and Zhao, X. (2000). The role of root border cells in plant defense. *Trends Plant Sci*. 5, 128-133.

Hernández, M., Dumont, M. G., Yuan, Q., and Conrad, R. (2015). Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. *Appl. Environ. Microbiol.* doi:10.1128/AEM.03209-14.

Hiltner, L. (1904). Über neuere Erfahrungen und Problem auf dem Gebiet der Bodenbakteriologie und unter besonderer Berücksichtigung der Gründüngung und Brachte. *Arb. Dt. Landwges.* 98, 59-78.

İnceoğlu, Ö., Salles, J. F., van Overbeek, L., and van Elsas, J. D. (2010). Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields. *Appl. Environ. Microbial.* 76, 3675-3684.

Jones, D. L., Hodge, A., and Kuzyakov, Y. (2004). Plant and mycorrhizal regulation of rhizodeposition. *New Phytol*. 163, 459-480.

Jones, D., Nguyen, C., and Finlay, D.R. (2009). Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil* 321, 5-33.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., *et al*. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6, 1378-1390.

Krüger, M., Frenzel, P., Kemnitz, D. and Conrad, R. (2005). Activity, structure and dynamics of the methanogenic archaeal community in a flooded Italian rice field. *FEMS Microbiol. Ecol*. 51, 323-33.

Krumböck, M., and Conrad, R. (1991). Metabolism of position-labelled glucose in anoxic methanogenic paddy soil and lake sediment. *FEMS Microbiol. Lett.* 85, 247-256.

Lu, Y., and Conrad, R. (2005). *In situ* stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309, 1088-1090.

Lu, Y., Rosencrantz, D., Liesack, W., and Conrad, R. (2006). Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. *Environ. Microbiol*. 8, 1351-1360.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Buchner, A., *et al*. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res*. 32, 1363-1371.

Lueders, T., and Friedrich, M. W. (2003). Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl. Environ. Microbiol.* 69, 320-326.

Lynch, J. M., and Whipps, J. M. (1990). Substrate flow in the rhizosphere. *Plant Soil* 129, 1-10. http://dx.doi.org/10.1007/BF00011685.

Oksanen, J., Blanchet, G. F., Kindt, R., Legendre, R., Minchin, P. R., O'Hara, R. B., *et al*. (2012). vegan: Community Ecology Package ver. 2.0-5.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789-799.

Pruesse, E., Peplies, J., and Glöckner, F. O. (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28, 1823-1829

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W.,Peplies, J., and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188-7196.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al*. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.*, gks1219. doi: 10.1093/nar/gks1219.

R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, Available online at: http://www.R-project.org/.

Read, D. B., Bengough, A. G., Gregory, P. J., Crawford, J. W., Robinson, D., Scrimgeour, C. M., *et al*. (2003). Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil. *New Phytol*. 157, 315-326.

Ren, G., Zhang, H., Lin, X., Zhu, J., and Jia, Z. (2014). Response of phyllosphere bacterial communities to elevated CO_2 during rice growing season. *Appl. Microbiol. Biotechnol.* 98, 9459-9471.

Rui, J., Peng, J., and Lu, Y. (2009). Succession of bacterial populations during plant residue decomposition in rice field soil. *Appl. Environ. Microbiol.* 75, 4879-4886.

Ryan, P. R., and Delhaize, E., (2001). Function and mechanism of organic anion exudation from plant roots. *Annu. Rev. Plant Physiol. Mol. Biol*. 52, 527-560.

Sangwan, P., Chen, X., Hugenholtz, P., and Janssen, P. H. (2004). *Chthoniobacter flavus* gen. nov., sp. nov., the first pure-culture representative of subdivision two, *Spartobacteria* classis nov., of the phylum *Verrucomicrobia*. *Appl. Environ. Microbiol.* 70, 5875-5881.

Shrestha, P. M., Noll, M., and Liesack, W. (2007). Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. *Environ. Microbiol.* 9, 2464-2474.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., *et al*. (2009). Introducing mothur: open-source, platform-independent, community-supported

software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537-7541.

Stubbs, V. E., Standing, D., Knox, O. G., Killham, K., Bengough, A. G., and Griffiths, B., (2004). Root border cells take up and release glucose-C. *Ann. Bot. Lond*. 93, 221-224.

Su, J. Q., Ding, L. J., Xue, K., Yao, H. Y., Quensen, J., Bai, S. J., *et al*. (2015). Long-term balanced fertilization increases the soil microbial functional diversity in a phosphorus-limited paddy soil. *Mol. Ecol.* 24, 136-150

Tomiyama, H., Ohshima, M., Ishii, S., Satoh, K., Takahashi, R., Isobe, K., *et al*. (2001). Characteristics of newly isolated nitrifying bacteria from rhizoplane of paddy rice. *Micro. Environ*. 16, 101-108.

Watanabe, T., Kimura, M., and Asakawa, S. (2006). Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). *Soil Biol. Biochem*. 38, 1264-1274. doi:10.1016/j.soilbio.2005.09.020

Watanabe, A., Takeda, T., and Kimura, M. (1999). Evaluation of origins of CH_4 carbon emitted from rice paddies. *J. Geophys. Res.* 104, 23623-23629.

Wegner, C. E., and Liesack, W. (2015). Microbial community dynamics during the early stages of plant polymer breakdown in paddy soil. *Environ. Microbial*. doi:10.1111/1462-2920.12815.

White, J. R., Nagarajan, N., and Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352. doi: 10.1371/journal.pcbi.1000352

Yanagi, M., and Yamasato, K. (1993). Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol. Lett.* 107, 115-120.

Yuan, Y., Conrad, R., and Lu, Y. (2009) Responses of methanogenic archaeal community to oxygen exposure in rice field soil. *Environ. Microbiol. Rep.* 1, 347-354.

Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., *et al*. (2003). *Gemmatimonas aurantiaca* gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum *Gemmatimonadetes* phyl. nov. *Int. J. Syst. Evol. Microbiol.* 53, 1155-1163.

Zhu, W., Lu, H., Hill, J., Guo, X., Wang, H., and Wu, W. (2014). ^{13}C pulse-chase labeling comparative assessment of the active methanogenic archaeal community composition in the transgenic and nontransgenic parental rice rhizospheres *FEMS Microbiol. Ecol*. 87, 746-756.

Chapter 3

Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage

Björn Breidenbach[1] and Ralf Conrad[1]*

[1]Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

Published in ***Frontiers in Microbiology*** (2015) 5:752. doi: 10.3389/fmicb.2014.00752

Contributions:

B.B. designed the study and the sampling scheme, performed sampling and all analysis, evaluated the data, performed statistical analysis and wrote the manuscript.

R.C. designed the study and the sampling scheme, wrote the manuscript.

3.1 Abstract

We studied the resident (16S rDNA) and the active (16S rRNA) members of soil archaeal and bacterial communities during rice plant development by sampling three growth stages (vegetative, reproductive and maturity) under field conditions. Additionally, the microbial community was investigated in two non-flooded fields (unplanted, cultivated with upland maize) in order to monitor the reaction of the microbial communities to non-flooded, dry conditions. The abundance of Bacteria and Archaea was monitored by quantitative PCR showing an increase in 16S rDNA during reproductive stage and stable 16S rRNA copies throughout the growth season. Community profiling by T-RFLP indicated a relatively stable composition during rice plant growth whereas pyrosequencing revealed minor changes in relative abundance of a few bacterial groups. Comparison of the two non-flooded fields with flooded rice fields showed that the community composition of the Bacteria was slightly different, while that of the Archaea was almost the same. Only the relative abundance of *Methanosarcinaceae* and Soil Crenarchaeotic Group increased in non-flooded versus flooded soil. The abundance of bacterial and archaeal 16S rDNA copies was highest in flooded rice fields, followed by non-flooded maize and unplanted fields. However, the abundance of ribosomal RNA (active microbes) was similar indicating maintenance of a high level of ribosomal RNA under the non-flooded conditions, which were unfavorable for anaerobic bacteria and methanogenic archaea. This maintenance possibly serves as preparedness for activity when conditions improve. In summary, the analyses showed that the bacterial and archaeal communities inhabiting Philippine rice field soil were relatively stable over the season but reacted upon change in field management.

3.2. Introduction

Methane (CH_4) is the second most important greenhouse gas after carbon dioxide (CO_2) and has a 25 times larger global warming potential than CO_2 (Forster *et al*., 2007). The global budget of atmospheric CH_4 is on the order of 500-600 Tg per year (Forster *et al*., 2007) and rice fields contribute in the range of 25-300 Tg CH_4 per year (Chen and Prinn, 2005, Bridgham *et al*., 2013). Rice production will probably increase, in order to feed an increasing human population (Van Nguyen and Ferrero, 2006) so that CH_4 emission from rice fields may also increase in future. In rice fields CH_4 is produced as end product of the anaerobic degradation of organic matter by a complex microbial community consisting of hydrolytic and fermentative bacteria, and methanogenic archaea (Zinder, 1993; Conrad, 2007). Flooded rice fields have been used as a model system for studying the functioning of anoxic methanogenic microbial communities (Conrad, 2007; 2009).

In rice fields methane is produced by two major physiological guilds, the acetotrophic and the hydrogenotrophic methanogens. Acetotrophic methanogens dismutate acetate to CH_4 and CO_2, while the hydrogenotrophic methanogens reduce CO_2 with H_2 to CH_4 (Conrad, 2007). Molecular characterization of 16S rDNA showed a worldwide distribution of methanogens in rice fields (China, Italy, Japan and Philippines) including *Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriales*, *Methanomicrobiales* and *Methanocellales* (Großkopf *et al*., 1998; Ramakrishnan *et al*., 2001; Wu *et al*., 2006). The composition of the soil archaeal community changes if temperature is increased (Peng *et al*., 2008; Conrad *et al*., 2009) or the rice field soil is treated with organic matter such as rice straw (Conrad and Klose, 2006; Peng *et al*., 2008). Under field conditions, however, the archaeal communities were usually found to be rather stable even after short term drainage or extended periods of managing rice fields as upland fields (Krüger *et al*., 2005; Watanabe *et al*., 2006; Fernandez Scavino *et al*., 2013). In a recent study of a Korean rice field, numbers of archaea and methanogens changed by less than a factor of two throughout a cropping season (Lee *et al*., 2014).

In contrast to the archaeal community it has been shown that the bacterial community in rice field soil changes with time after flooding (Noll *et al*., 2005; Rui *et al*., 2009). Bacterial communities in irrigated rice fields are described as complex (Asakawa and Kimura, 2008) and differ between oxic and anoxic zones (Shrestha *et al*., 2007).

Additionally, temporal and spatial changes in the composition of the bacterial communities with changing soil conditions were observed (Noll *et al*., 2005; Shrestha *et al*., 2009). Variations in relative abundance of dominant phyla under alfalfa-rice crop rotation system were revealed (Lopes *et al*., 2014) whereas pasture-rice crop rotation showed a rather stable bacterial community composition (Fernandez Scavino *et al*., 2013).

Moreover, archaeal and bacterial communities in the rhizosphere can be shaped by the plant species (e.g. Grayston *et al*., 1998; Smalla *et al*., 2001; Conrad *et al*., 2008). Several other studies demonstrated that plant type had an effect on soil microbial community structure (Marschner *et al*., 2001; Smalla *et al*., 2001; Costa *et al*., 2006). In addition to plant residues and soil organic matter, rhizodeposits are the major substrate input into soil (Kimura *et al*., 2004). Rhizodeposits are plant-derived carbon-containing compounds, which are actively secreted via the plant roots or originate from sloughed-off root cells (reviewed by Dennis *et al*., 2010). Rhizodeposition takes place at the zone around the plant root called rhizosphere which was shown to harbor a specific microbial community (Kowalchuk *et al*., 2010). Rhizodeposition depends on environmental factors, plant species, type and cultivar as well as plant age (Aulakh *et al*., 2001; Uren, 2007). The microbial community in the rhizosphere may be influenced by these variations in rhizodeposition.

Therefore, we hypothesized that the microbial community in rice field soil will be influenced by rice plant growth stage. Since a comprehensive seasonal record of resident and active microorganisms was lacking, we investigated the archaeal and bacterial community in the soil under field conditions by sampling three distinct plant growth stages. Additionally, the microbial community was investigated in two fields that were not flooded and were either unplanted or cultivated with upland maize in order to monitor the reaction of the rice specific microbial community to non-flooded conditions and to the presence or absence of maize. The microbial composition and abundance was assessed by fingerprinting with terminal-restriction fragment length polymorphism (T-RFLP) and quantitative PCR (qPCR) targeting the archaeal and bacterial ribosomal 16S rRNA and 16S rDNA, respectively. In order to identify changes in the lower taxonomic groups, archaeal and bacterial 16S rRNA was targeted by 454 pyrosequencing. Interestingly, we observed rather stable archaeal and bacterial communities in the soil during rice plant growth but detected more pronounced differences between flooded and non-flooded fields.

3.3 Material and methods

3.3.1 Sampling site and sample processing

The sampling site was located at the International Rice Research Institute (IRRI) in Los Banos, Philippines. Detailed site description can be found in Heinz *et al*. (2013). Briefly, we studied fields cultivated with irrigated rice throughout one cropping season at the vegetative (February 2012), reproductive (March 2012) and maturity (May 2012) growth phase of the rice plants (variety: NSIC Rc222). Additionally rice fields, which had been drained and were now managed as upland fields cultivating upland maize (variety: Pioneer P3482YR) were sampled after plowing and before maize seeding and fertilization as unplanted and drained rice field (unplanted) and during the reproductive growth phase of maize (maize). The study site was cropped with paddy rice in both wet and dry season over two decades (Weller *et al*., 2014). This season (dry 2012) was the first season in which the fields were managed as upland maize fields. Fields were operated in triplicates and managed with conventional N-fertilization (rice: seeding 30 kg N/ha, 30 kg P_2O_5/ha, 30 kg K_2O/ha; at 28 and 55 days after seeding (DAS) 50 kg N/ha; maize: seeding 30 kg N/ha, 50 kg P_2O_5/ha, 30 kg K_2O/ha; at 27-29 and 47-50 DAS 50 kg N/ha). In each of these fields we randomly selected three sampling plots of one square meter and sampled one soil core (5 cm diameter) from each plot. Soil cores were always taken from the vicinity of a plant (ca. 10 cm). The soil contained numerous fine roots and thus was most probably influenced by the plant roots. However, no attempts were made to separate a specific rhizospheric soil compartment. Subsequently, soil samples of 5 g were taken from the middle of the core (~ 10 cm depth), added to 10 mL RNAlater© solution (Life Technologies, Darmstadt, Germany), kept on ice and later stored at -20°C to ensure RNA stability. For further analysis (determination of soil variables), additional samples of 50 g were taken from the same soil core and stored at -20°C.

3.3.2 Determination of soil variables

For determination of soil water content small amounts of soil (1-5 g) were dried at 65°C for 3 days. The gravimetric water contents of the samples from fields cultivated with rice or maize and unplanted fields were 42.8 ± 3.5%, 34.3 ± 1.2% and 36.0 ± 2.2%,

respectively. The pH of the soil was analyzed following the DIN ISO 10390 protocol. Briefly, 3 g of soil was mixed with 0.01 M $CaCl_2$ in a ratio of 1:2.5 and incubated rotating at 25°C for 10 min. Subsequently, the samples were incubated at 25°C for 60 min without agitation and then, after shaking the sample, the pH was measured using a pH meter (pH530 WTW, Weilheim, Germany). The pH values in rice, maize and unplanted fields were pH 6.8, 6.6 and 6.2, respectively. The soil texture was silt loam and the determination was conducted using a laser particle measuring device (LS13320, Beckmann-Coulter, Krefeld, Germany) at the geographic institute of the RTWH Aachen.

3.3.3 Nucleic acid extraction

Nucleic acids were extracted following a modified version of the protocol from Bürgmann *et al.* (2001). Briefly, after removal of RNAlater© solution by centrifugation at 2,500 x g for 2 min, 0.5 g of soil were extracted via bead-beating for 45 s at 6 m/s using a FastPrep®-24 (MP Biomedicals, Eschwege, Germany) in the presence of a 850 µl extraction buffer (20 ml 1 M sodium phosphate (pH 8.0), 2.5 g SDS, 10 ml 0.5 M EDTA (pH 8.0) and 2 ml 5 M NaCl). The tube was centrifuged at maximum speed for 5 min at 20°C. Then, 850 µl of phenol/chloroform/isoamylalcohol (25:24:1; Fluka, Sigma-Aldrich, Taufkirchen, Germany) was added to the supernatant and mixed. The bead beating was repeated twice using fresh extraction buffer. After mixing, the tubes were centrifuged for 5 min at maximum speed at 20°C. Then, 800 µl of chloroform/ isoamylalcohol (24:1; Fluka, Sigma-Aldrich, Taufkirchen, Germany) was added to the supernatant. After further centrifugation, 1 ml of precipitation solution (20 g PEG 6000, 16.6 g NaCl) was added to the aqueous supernatant, mixed, and kept at room temperature for 1 h. After centrifugation for 1 h with maximum speed at 4°C the sample was resuspended in 75% ice cold ethanol (Roth, Karlsruhe, Germany) and subsequently centrifuged for 10 min at maximum speed and 4°C. The resulting pellet was air dried and resuspended in 100 µl nuclease free water (Invitrogen, Darmstadt, Germany) and stored at -80°C until analysis. The total nucleic acids in 50 µl aliquot were digested with 37.5 µl nuclease free water (Invitrogen), 2.5 µl RNase-free DNase and 10 µl buffer RDD (Qiagen, Hilden, Germany) at room temperature for 10 min. The digest was then purified using RNeasy kit (Qiagen) following the RNA Cleanup protocol in the manufacturer´s instructions. Complete DNA removal was verified by failure

to obtain a PCR amplification product of bacterial 16S rDNA with the purified RNA template using the conditions described below. cDNA was synthesized from purified RNA using SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Random hexamer primers (50ng/µl) were used for complete cDNA synthesis which was used for amplification of the archaeal and bacterial 16S rRNA.

3.3.4 Quantitative polymerase chain reaction

The quantification of archaeal and bacterial 16S rDNA/rRNA was conducted using quantitative polymerase chain reaction (qPCR) using primer combinations Ba519f / Ba907r (Stubner, 2002) for bacterial and Ar364f (Burggraf *et al*., 1997) / Ar934br (Großkopf *et al*., 1998) for archaeal genes. The qPCR was conducted in 96-well micro titer plates (BioRad, München, Germany) using an iCycler MyiQ™ (BioRad). Each qPCR reaction contained in a total volume of 25 µl, 1 x SYBRGreen Ready Mix (Sigma), 3 mM $MgCl_2$ (Sigma), 0.25-0.66 µM of each primer and 1 µM FITC (fluorescein thiocyanat; BioRad) as well as 1-2 µl target DNA respectively cDNA. Purity of the used reagents was ensured using negative controls not containing any DNA matrix. The DNA standard prepared from clones containing bacterial or archaeal 16S rDNA in a plasmid insert was applied in a dilution series containing 1 x 10^7 to 1 x 10^1 gene copies. The thermal profile used for amplification included 40 to 50 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C (Ba519f /Ba907r) or 66°C (Ar364/Ar934br) for 20-30 s and primer extension at 72°C for 45 s. Afterwards a melting curve from 75°C to 95°C (0.2°C s^{-1}) was performed in order to confirm specificity of the real time PCR reaction. The data were analyzed using BioRad IQ5 2.0 Standard Edition Optical System software (Biorad).

3.3.5 Terminal fragment length polymorphism (T-RFLP)

T-RFLP analysis of archaeal and bacterial 16S rRNA/rDNA was conducted based on fractionation of terminal fluorescence-labeled PCR products after use of restriction enzymes as described (Chin *et al*., 1999) using the primer combination Ar109f (Großkopf *et al*., 1998) / Ar912rt-FAM (Lueders and Friedrich, 2003) and Ba27f-FAM (Osborne *et al*., 2005) / Ba907r (Muyzer *et al*., 1995), respectively. All PCR reactions were performed in a total

volume of 50 µl. For amplification each reaction contained 5 x Green GoTaq® Flexi buffer (Promega, Mannheim, Germany), 200 µM deoxy-nucleoside triphosphates (dNTPs; Fermentas, St. Leon-Rot, Germany), 0.5 µM of each primer, 10 µg bovine serum albumin (BSA; Roche, Grenzach, Germany), 1 U GoTaq® Flexi DNA polymerase (Promega) and 1 µl DNA matrix (in most cases diluted to a concentration of 20 ng/µl). All amplifications were carried out in a GenAmp 9700 Thermocycler (Applied Biosystems, Carlsbad CA, USA). The thermal profile used for amplification included 25 to 30 cycles of primer annealing at 52°C for 45 s, primer extension at 72°C for 90 s, and denaturation at 94°C for 45 s. PCR product purification was conducted using the GenElute™ PCR Clean-up kit (Sigma) following the manufacturer´s instructions. The purified amplicons were digested by using *MspI* (cutting side: 5'-C▼CGG-3`, 37° C; Fermentas) for bacterial and *TaqI* (cutting side: 5'-T▼CGA-3`, 65° C; Fermentas) for archaeal 16S rDNA/rRNA. The fragmented DNA was purified using SigmaSpin™ Post Reaction Clean-Up columns (Sigma) following the manufacturer´s instructions. T-RFLP reactions contained 0.2 µl size standard (X-rhodamine MapMarker® 1000, BioVentures, Murfreesboro, USA). Separation was accomplished using capillary electrophoresis in an ABI PRISM 3130 Genetic Analyzer (Applera). Data analysis was conducted using GENESCAN Analysis software 4.0 (Applied Biosystems, Carlsbad, USA). Normalization and standardization of the T-RFLP profiles was done according the method from Dunbar *et al*. (2001). The relative abundance was calculated from the ratio between the height of the fluorescence signal and the total height of all signals in one sample.

3.3.6 Cloning and sequencing

A clone library of archaeal 16S rDNA was created for subsequent phylogenetic classification. The pGEM®-T Easy Vector System (Promega) was used. The purified PCR products were ligated into the pGEM®-T Easy Vector according to the manufacturer´s instructions. Each ligation reaction contained 5 µl 2X Rapid Ligation Buffer (Promega), 1 µl pGEM®-T Easy Vector (50 ng; Promega), 1 µl T4 DNA ligase (3 Weiss units/µl; Promega) and 50 ng PCR product. Sterile water was added to reach a total volume of 10 µl. The transformation of *Escherichia coli* JM109 high efficient competent cells (Promega) was carried out according to the manufacturer´s instructions. Randomly chosen white colonies

were sequenced using Sanger sequencing. The received raw data (electropherograms) were processed using the program Seqman II (DNAStar, Madison, USA). The phylogeny of the archaeal sequences was analyzed using the ARB software (http://www.arb-home.de/). For the archaeal 16S rRNA sequences the public available database was downloaded from SILVA homepage (http://www.arb-silva.de/) and integrated into ARB. Alignment was conducted using the Fast Aligner tool in ARB (Ludwig *et al*., 2004). The alignment was then manually checked and where necessary corrected. Subsequently, the aligned sequences were calculated into the archaeal 16S rRNA tree under usage of the neighbor-joining algorithm as described in detail by Wu *et al*. (2006). The restriction sides characteristic for the fragment length of the T-RFs were determined. The T-RFs determined from T-RFLP analyses were assigned to the corresponding clones and their phylogeny. The archaeal 16S rRNA sequences data have been submitted to the GenBank databases under accession numbers: KM463011 - KM463082.

3.3.7 454 Pyrosequencing

Tagged pyrosequencing of the bacterial and archaeal community was conducted using primer combinations F515/R806 (Bates *et al*., 2011) and Arch344F (Casamayor *et al*., 2002) / A934br (Großkopf *et al*., 1998), respectively. The forward primers were tagged with a unique 8-base pair barcode. Sequencing of the PCR products was done at the Max Planck Genome Centre in Cologne using a Roche 454 Genome Sequencer GS FLX+. Data analysis was performed using mothur software package version 1.31.2 (http://www.mothur.org/) following the standard operational procedure including sequence quality management (SOP, Schloss *et al*., 2009). OTU clustering and analysis was conducted using UPARSE pipeline as described by Edgar (2013). Only microbial high-quality sequences with a minimum read length of 200 bp were used. Sequences that did not match the primer sequences and were smaller than 200 bp or contained any ambiguities were excluded from further analysis. After denoising, sequences were aligned against the SILVA bacteria/archaea 16S rDNA database (Schloss *et al*., 2011; Pruesse *et al*., 2007). Sequences which were not assigned to bacteria or respectively archaea were discarded. Operational taxonomic units (OTU) were defined using a distance matrix with 3% dissimilarity (Zinger *et al*., 2011). Further analyses including rarefaction curves, species richness and diversity indices were conducted as described in the

SOP pyrosequencing pipeline (Schloss *et al*., 2011). An overview of the number of sequences retrieved and the accession numbers of the submitted sequences can be found in Tables 3.2 and 3.3.

3.3.8 Statistical analysis

Statistical analyses were done in R version 2.14.1 (R Development Core Team, 2011). If necessary, normal distribution was achieved by log-transforming the data. Analysis of variance (ANOVA), PERMANOVA (ADONIS) and canonical correspondence analysis (CCA) were conducted with package vegan version 2.0.5 (Oksanen *et al*., 2012). All levels of significance were defined at $P \leq 0.05$. Ternary plots were created using package vcd version 3.0.3.

3.4 Results

3.4.1 Bacterial and archaeal 16S rDNA/rRNA copy numbers

For quantification of bacteria and archaea in rice field soil during rice plant growth we used quantitative PCR (qPCR) targeting the bacterial and archaeal 16S ribosomal RNA (16S rRNA) and their genes (16S rDNA). Copy numbers of bacterial and archaeal 16S rDNA and rRNA were quantified at three different growth stages and in differently cropped fields (Figure 3.1). Both bacterial and archaeal 16S rDNA copy numbers were highest during rice growth at reproductive stage, whereas the 16S rRNA copy numbers were constant during the whole season (Figure 3.1A, B). Comparing rice and maize cultivated soils during the reproductive growth phase, the highest copy numbers of 16S rDNA and rRNA were detected in the rice fields (Figure 3.1C, D). The unplanted fields contained less 16S rDNA copies than the fields cultivated with either rice or maize (Figure 3.1C, D). However, the numbers of archaeal and bacterial 16S rRNA copies were similar to those in the rice fields (Figure 3.1C, D) resulting in a high ratio of rRNA and rDNA copies (Figure 3.2). In contrast, bacterial 16S rRNA copies were lower in the maize field than in the rice cultivated and unplanted fields (Figure 3.1C).

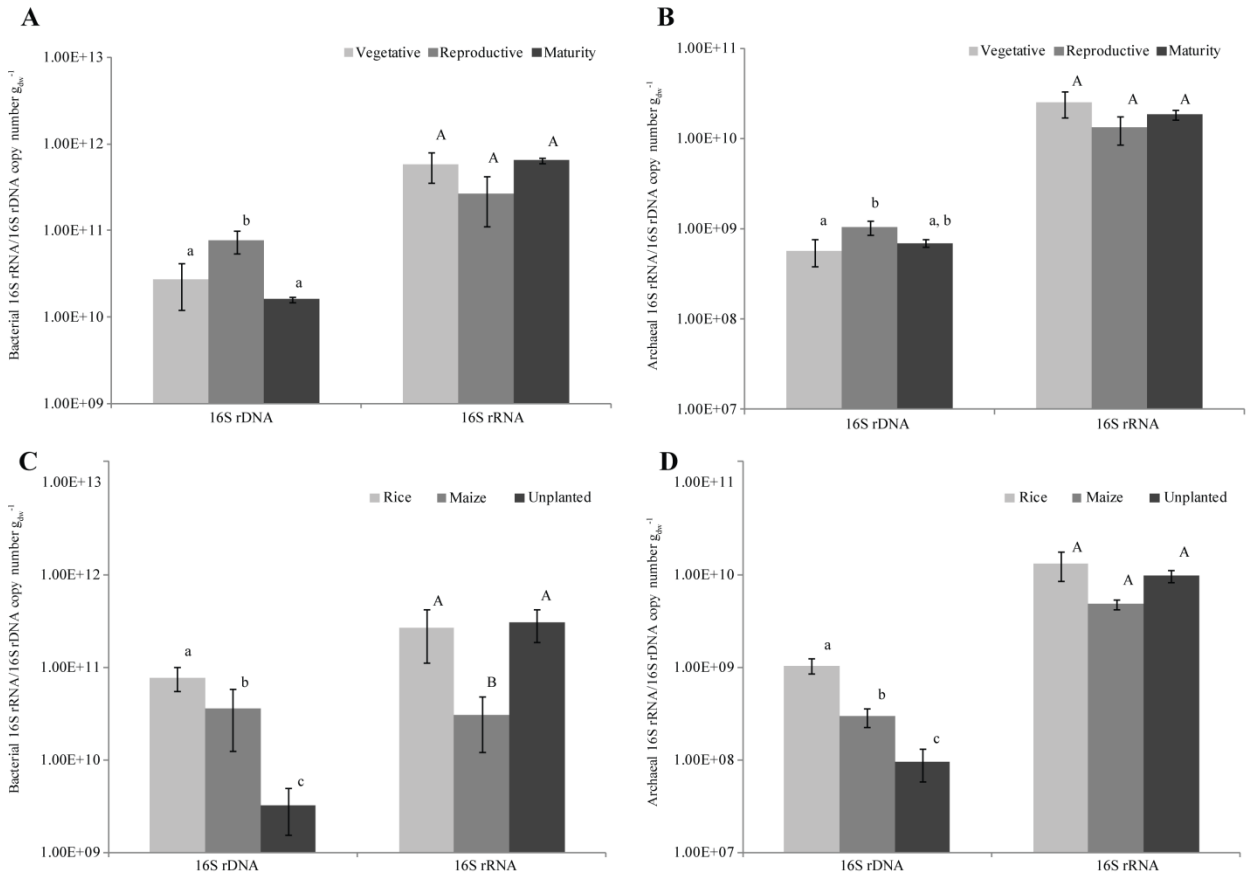


Figure 3.1 Ribosomal 16S rRNA and rDNA copy numbers quantified using qPCR. Abundance of bacterial 16S rDNA and rRNA (**A, C**) and archaeal 16S rDNA and rRNA (**B, D**) in rice fields at different plant growth stages (**A, B**) as well as in fields planted with rice, maize or unplanted at the reproductive growth stage (**C, D**). Different letters indicate significant difference (mean ± SE, n=9).

Although the ratio of bacterial and archaeal rRNA/rDNA copies was significantly increased in unplanted fields in comparison to the fields cultivated with either rice or maize across all the replicates sampled, samples from replicate field 7 did not show such increase (Figure 3.2). The behavior of these particular replicates could not be explained by analyzing possible correlation with soil characteristics (contents of carbon, nitrogen, sulfate, nitrate, water).

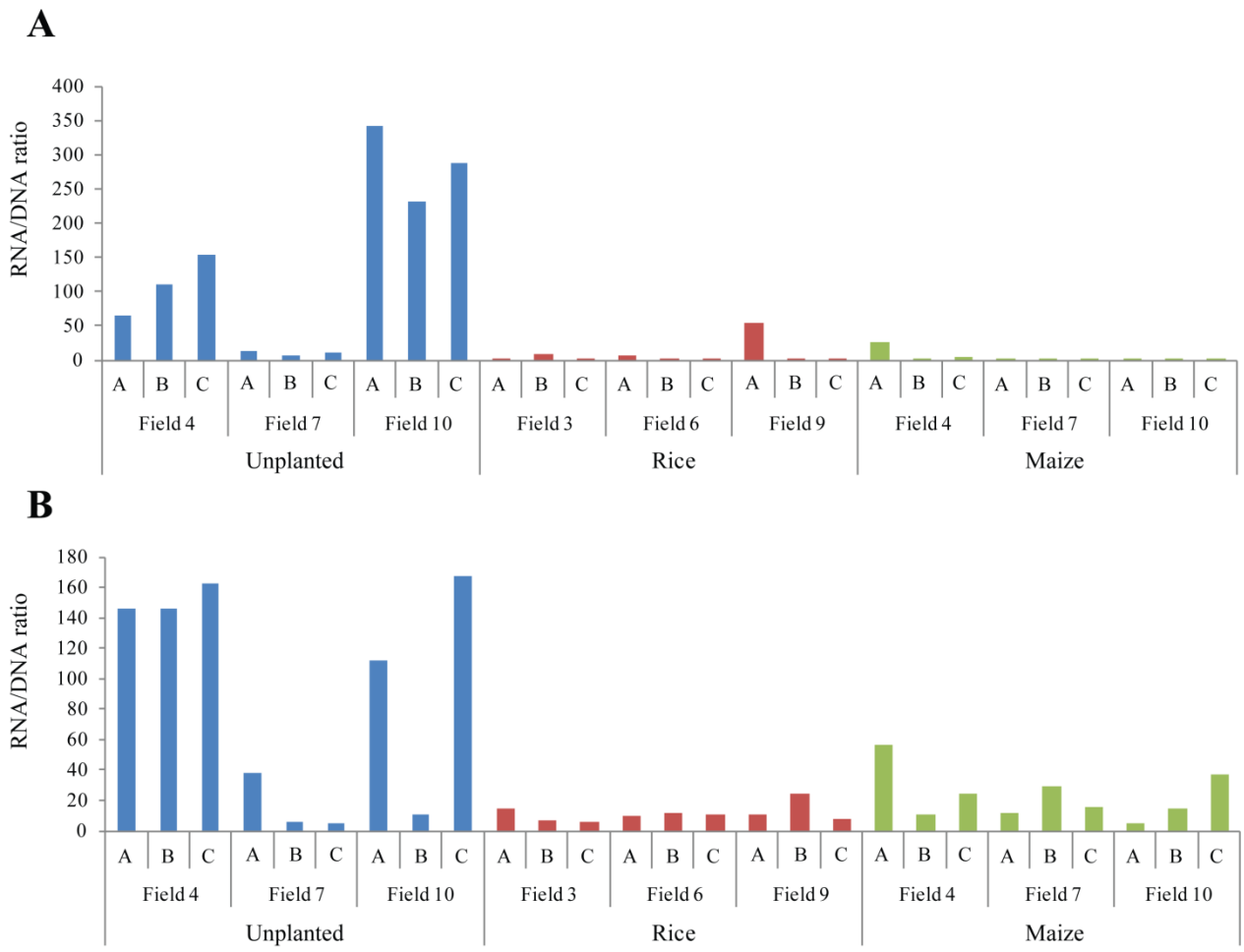


Figure 3.2 Ratio of ribosomal 16S rRNA and rDNA copy numbers quantified using qPCR. Bacterial **(A)** and archaeal **(B)** ratios are shown for each replicate in the unplanted, rice and maize cultivated fields.

3.4.2 Bacterial and archaeal community analyzed by T-RFLP

For community profiling we used T-RFLP targeting the bacterial and archaeal 16S rDNA and rRNA. To identify parameters which significantly explain the variance in the microbial community, canonical correspondence analysis (CCA) was performed. Field management (rice, maize, unplanted), growth stage (vegetative, reproductive, maturity) and gravimetric water content were identified to significantly affect the microbial community explaining 6-23% of the variance (Figure 3.3A-D). The pure effect of each factor is shown in Supplement Table 3.1, with field management explaining 12-16%, growth stage 11-23% and gravimetric water content 6-12% of the variance. Although these factors were significant, the resident bacterial (Supplement Figure 3.1) and the archaeal (Figure 3.4) community composition did not change significantly during rice plant growth (ADONIS, $P > 0.05$). The non-flooded fields (unplanted and maize) also revealed a bacterial and archaeal community composition that was not significantly different from the rice field community (ADONIS, $P > 0.05$). Only the archaeal T-RF of 186 bp significantly increased during maize cultivation in comparison to the rice fields (Figure 3.4; ANOVA, $P < 0.05$). The more active bacterial and archaeal communities (16S rRNA) showed only minor variations in relative abundance of T-RFs during rice plant growth and in the unplanted and maize fields. These variations were not statistically significant (ADONIS, $P > 0.05$).

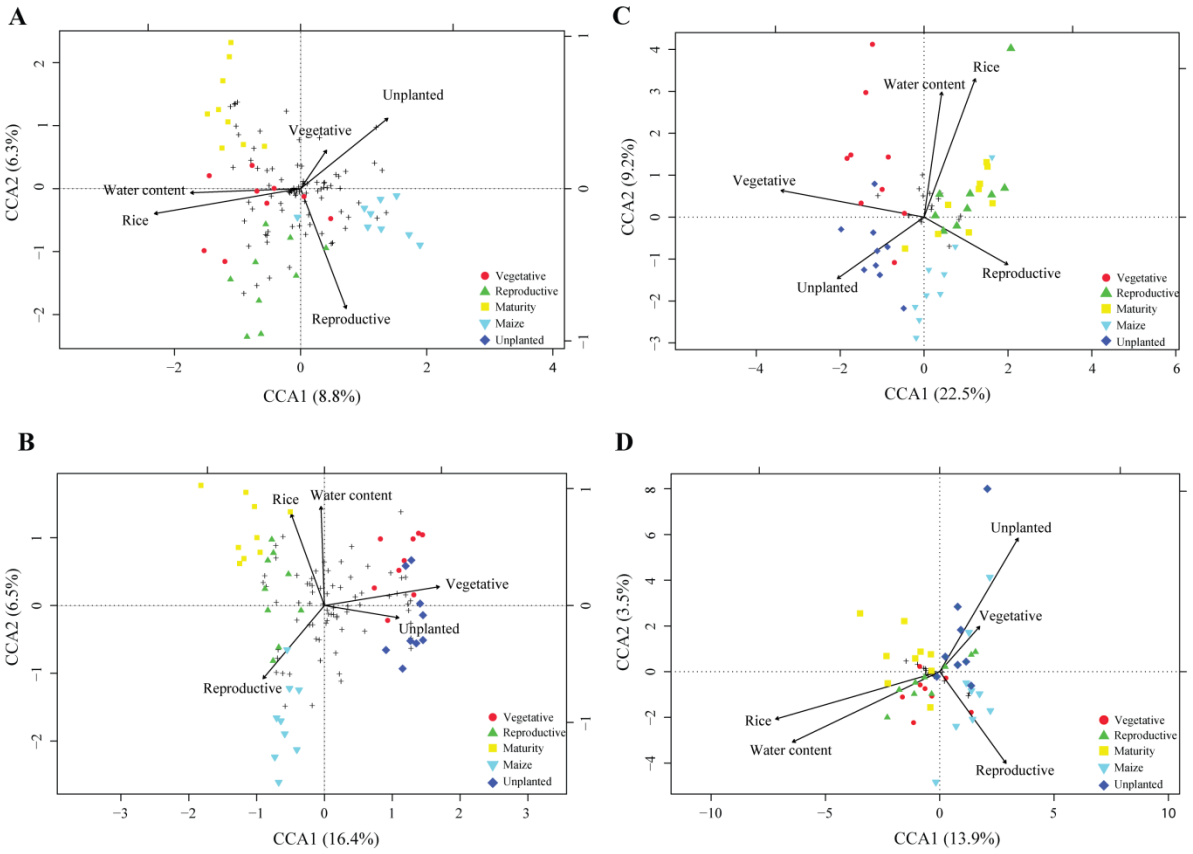


Figure 3.3 Canonical correspondence analysis (CCA) biplot of T-RFLP based on bacterial and archaeal communities. T-RFLP based communities of bacterial 16S rDNA and rRNA **(A,B)** as well as archaeal 16S rDNA and rRNA **(C,D)** are displayed. Arrows indicate the direction and relative importance (arrow lengths) of environmental variables associated with bacterial and archaeal community structures, respectively. Solely the environmental variables significantly influencing the model were displayed (ANOVA $p < 0.05$). Circle, triangle and square symbols, respectively, represent vegetative, reproductive and maturity growth phase of rice. Inverted triangle and diamante symbols, respectively, characterize samples originating from maize and unplanted fields while crosses represent T-RFs.

The archaeal T-RFs were assigned to different archaeal lineages by sequence analysis (Table 3.1). The assignment was based on a clone library of 16S rDNA containing 72 randomly selected clones retrieved from soil cultivated with rice and maize. The major T-RFs of 95, 186, 286, 396 and 810 bp were assigned as *Methanobacteriales*, *Methanosarcinaceae*, *Methanosaetaceae*, *Methanocellales* and Miscellaneous *Crenarchaeota*, respectively (Table 3.1). Some additional T-RFs of minor relative abundance were detected at 75, 308, 611, 671, 682, 695, 737 and 771 bp (most of them are too minor to be shown in Figure 3.4), which were not represented in the clone library, and therefore could not be assigned. A few clones, which were solely found in the clone library but not in the T-RFLP analysis, were assigned as Miscellaneous *Crenarchaeota* (97, 263, 333, 656. 725 bp).

Table 3.1 Lengths of distinct terminal restriction fragments (T-RFs) of different archaeal 16S rDNA clones obtained rice and maize cultivated Philippine rice field soil and affiliation with a distinct phylogenetic lineage by 16S rDNA sequence analysis of the clones.

Phylogenetic affiliation	*Terminal restriction fragment lenght (bp)*	*No. of clones rice field*	*No. of clones maize field*
Miscellaneous *Crenarchaeota*	83, 95, 191, 254, 263, 286, 379, 395, 656, 725, 795, 810	27	28
Soil Crenarchaeotic Group	191	-	1
Crenarchaeota Group C3	380	-	1
Methanobacteriales	95	1	-
Methanosarcinaceae	186	9	2
Methanosaetaceae	286	1	-
Methanocellales	396	1	1

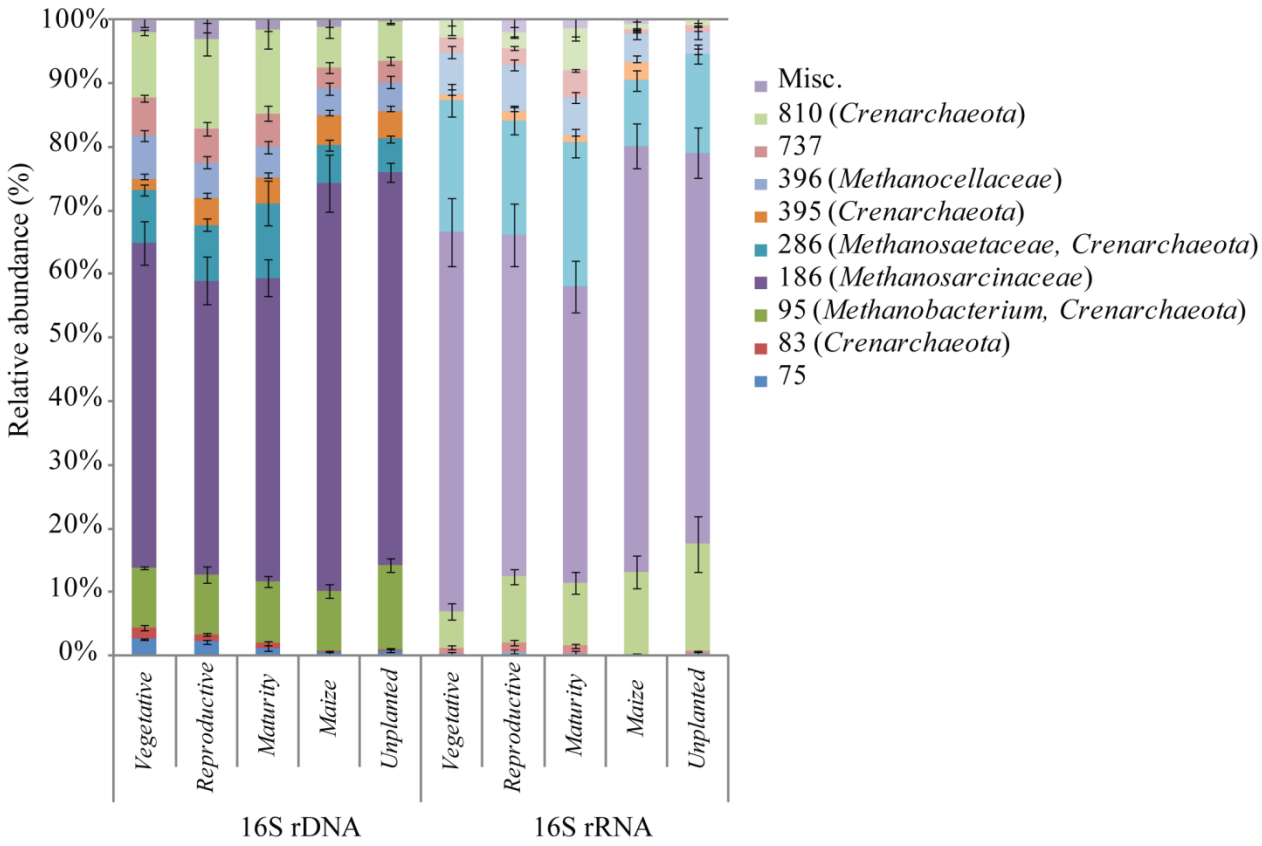


Figure 3.4 Histograms of the relative abundance of T-RFs obtained from T-RFLP analysis of archaeal 16S rDNA and rRNA during rice plant growth. Terminal restriction fragment sizes and affiliated clone taxonomy are given in brackets. Bars represent standard errors of n=9.

3.4.3 Pyrosequencing of bacterial 16S rDNA and rRNA

Pyrosequencing targeting the bacterial 16S rDNA and rRNA was conducted in order to indentify the resident and the active bacterial phylotypes in the Philippine rice field soil and to monitor the influence of plant growth stage on the bacterial community composition. Therefore, triplicate samples were sequenced for each growth stage resulting in 3,468 to 11,311 high quality sequences of rDNA as well as 2,062 to 10,275 sequences of rRNA (Table 3.2). For bacterial rDNA, the most dominant phylum was *Proteobacteria* (23-32%) followed by *Acidobacteria* (16-20%). Other important bacterial phyla were *Chloroflexi* (8-10%), *Verrucomicrobia* (5-6%), *Firmicutes* (4-5%), *Actinobacteria* (2-3%), *Planctomycetes* (2%) and *Cyanobacteria* (1-3%) (Supplement Figure 3.2A - E). The bacterial community composition did not change dramatically during the rice growing season (Supplement Figure 3.2A - C). Comparison of the dominant OTUs retrieved at different rice plant growth stages showed a uniform distribution over the season (Figure 3.5A). Only OTUs with a minor relative abundance were distinct for a particular growth stage, e.g., OTU 396 identified as *Anaeromyxobacter*, which was only found at the vegetative growth stage (Figure 3.5A). Comparison of unplanted, maize and rice cultivated fields showed more pronounced differences among bacterial OTUs. The OTUs number 1 (*Spartobacteria*), 4 (Unclassified) and 7 (*Acidobacteria Gp25*) were more abundant in the unplanted and in the upland maize fields than in the rice fields, while OTU number 9 (*Deltaproteobacteria*) was relatively more abundant in the rice fields (Figure 3.5C). Additionally, unplanted fields as well as fields cultivated with upland maize showed significantly less *Proteobacteria* in comparison to rice fields (Supplement Figure 3.2A, D, E). The lower relative abundance of the *Proteobacteria* was due to the lower abundance of *Geobacteraceae*. Otherwise, however, the bacterial community composition was not affected by the rice growth stage or the type of crop (ADONIS, $P > 0.005$).

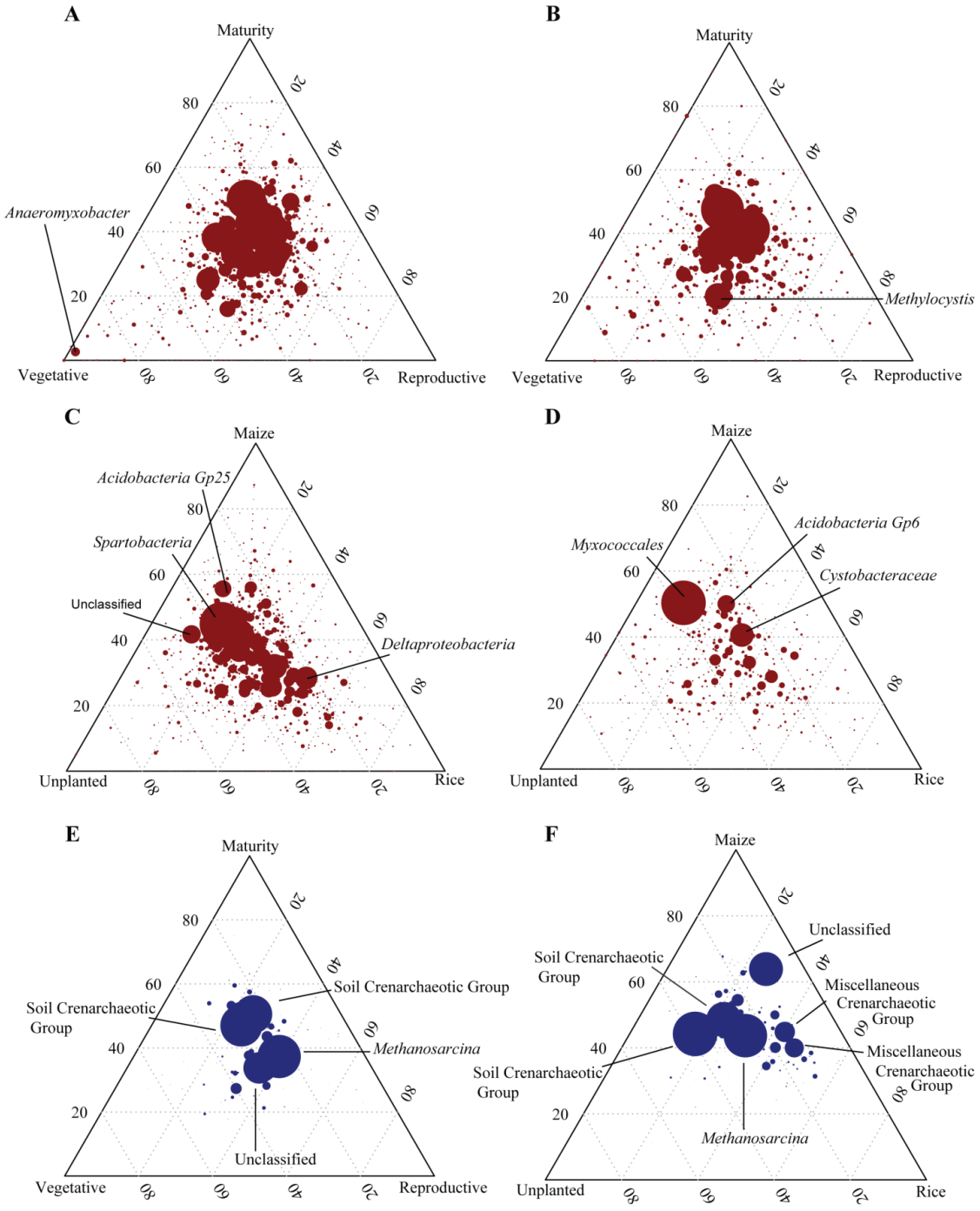


Figure 3.5 Ternary plots showing the distribution of bacterial and archaeal 16S rDNA/rRNA based OTUs. Axes represent rice plant growth stages (vegetative, reproductive and maturity) **(A, B, E)** as well as unplanted and maize cultivated fields in comparison to rice cultivated fields **(C,D,F)** and the percentage of reads associated with each sample for each OTU. Bacterial 16S rDNA **(**red, **A, C)** and 16S rRNA **(B, D)** as well as archaeal 16S rDNA **(**blue, **E, F)** are displayed. Each circle represents an

individual OTU while its size indicates number of reads associated. The position of each OTU is determined by the contribution of the sample type to the total count (n=3).

The sequences of ribosomal RNA presumably represent the more active bacterial community. This community was composed of the same phyla as the ribosomal gene-based community, but exhibited a different composition (Supplement Figure 3.2F - J). The most dominant phyla within the bacterial rRNA community were *Proteobacteria* (36 -40%) followed by *Acidobacteria* (14-18%). Other important bacterial phyla were *Chloroflexi* (3-4%), *Verrucomicrobia* (4-6%), *Firmicutes* (3-4%), *Actinobacteria* (2-4%), *Planctomycetes* (5-6%) and *Cyanobacteria* (3-9%) (Supplement Figure 3.2F - J). At the phylum level the bacterial community was not significantly different between the different plant growth stages (ADONIS, P> 0.005). Nevertheless, specific bacterial groups changed in abundance during rice plant growth. Only OTUs with relatively low abundance were characteristic for individual growth stages, whereas the dominant OTUs were equally distributed and observed at all three growth stages. Only OTU number 4 (*Methylocystis*) was more prominent at the vegetative and reproductive than at the maturity stage (Figure 3.5B). Additionally, *Verrucomicrobia* and *Bacteroidia* increased from vegetative to reproductive growth stage, *Cyanobacteria* decreased (data not shown). Comparison of the ribosomal OTUs in unplanted, maize and rice-cultivated soils showed more pronounced preferences (Figure 3.5D). The most dominant OTU number 1 (*Myxococcales*) was preferentially associated with the non-flooded fields (unplanted, maize) while OTU number 3 (*Acidobacteria Gp6*) and 2 (*Cystobacteraceae*) were found in all field types. Additionally unplanted fields showed higher *Bacteroidetes* and *Sphingobacteria* than the rice fields (ANOVA, p< 0.05). Less *Verrucomicrobia* were detected in the unplanted fields in comparison with rice and maize fields.

The analysis of presence and absence of individual OTUs based on rRNA revealed that the OTUs detected in all fields (core OTUs) constituted 70%, 74% and 71% of the relative abundance in rice, maize and unplanted fields, respectively. Direct comparison of the rice and the unplanted fields showed similar distribution of bacterial lineages in core, shared and unique OTUs (Figure 3.6). The relative abundance of core OTUs assigned as *Deltaproteobacteria* and unique OTUs assigned as *Armantimonadetes*, *Bacteroidetes*,

Alphaproteobacteria and *Gammaproteobacteria* was increased in the unplanted fields (Figure 3.6).

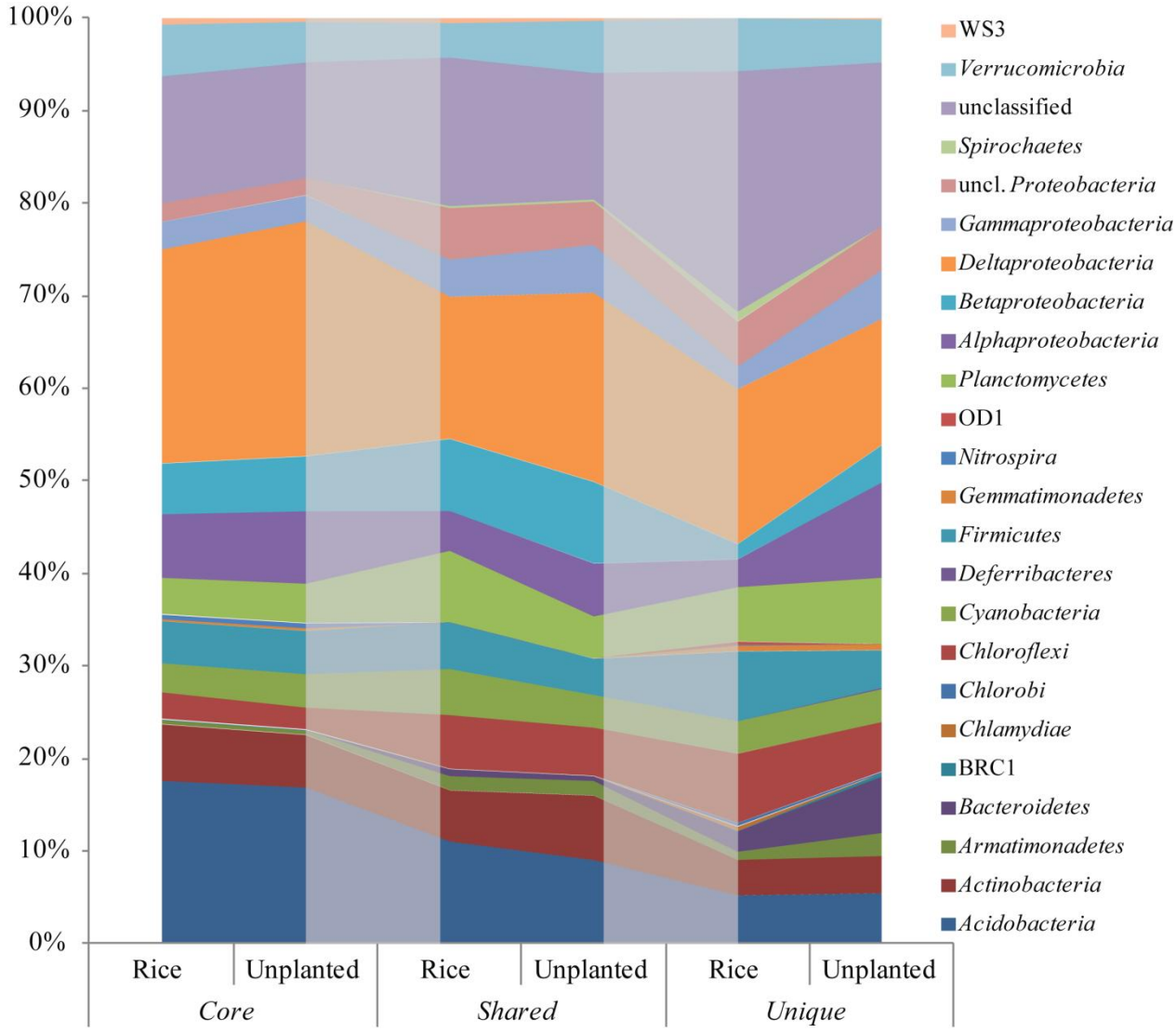


Figure 3.6 OTU based relative sequence abundance of bacterial phyla based on 16S rRNA in the rice and unplanted fields. OTUs detected in all fields (core), only in unplanted and rice cultivated fields (shared) and OTUs only detected in rice or unplanted fields (unique) were presented. The shaded areas serve visualization and have no special meaning.

3.4.4 Pyrosequencing of archaeal 16S rDNA and rRNA

Analogously to the bacterial community, the archaeal community was analyzed using pyrosequencing targeting archaeal 16S rDNA and rRNA. Sequencing resulted in 1,326 to 9,290 high quality sequences of rDNA as well as 532 to 1,962 sequences of rRNA (Table 3.3). For archaeal rDNA, the major taxa with a relative abundance of >2% in at least one sample are shown in Supplement Figure 3.3A. The most dominant class was *Methanomicrobia* (40-50% relative abundance) followed by Soil Crenarchaeotic Group (20-34%), Misc. Crenarchaeotic Group (17-23%) and *Methanobacteria* (2-6%). The class *Methanomicrobia* was further subdivided into orders and families. The most dominant archaeal order was *Methanosarcinales* with the families *Methanosarcinaceae*, *Methanosaetaceae* and the group GOM Arc I (Supplement Figure 3.3A). Comparison of the dominant OTUs retrieved at different rice plant growth stages showed a uniform distribution over the season (Figure 3.5E). Most dominant OTUs were assigned as Soil Crenarchaeotic Group and *Methanosarcina* and their distribution did not change in composition during rice plant growth (Figure 3.5E). Several trends, albeit not significant, are worth mentioning. Thus, the relative abundance of *Methanobacteriaceae*, *Methanosarcinaceae*, *Methanosaetaceae* and *Methanocellaceae* was relatively high in the reproductive stage, while that of GOM Arc I was relatively low (Supplement Figure 3.3A). The relative abundance of the genera *Methanolinea* and Candidatus *Methanoregula* decreased from vegetative to later growth stages.

Comparison of unplanted, maize and rice cultivated fields showed again a uniform distribution of the OTUs (Figure 3.5F). However, the OTUs assigned as Misc. Crenarchaeotic Group and unclassified were more abundant in the planted fields (rice, maize), while the unclassified OTU was more associated with the maize field. Similar to T-RFLP analysis an increase of *Methanosarcinaceae* in the maize fields and unplanted fields was observed, but not statistically significant (Supplement Figure 3.3A). In fact, no significant changes in the archaeal community composition were observed (ADONIS, $P > 0.05$).

Table 3.2: Number of bacterial sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047272 for bacterial sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Gene	*Name*	*Growth Stage*	*Plant*	*Barcode*	*Accession*	*Raw seqs*	*No. seqs**	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*
	RWVF3	Vegetative	Rice	ACGTAC	SRS715481	9579	6472	2089	0.83	3794	585
	RWVF6	Vegetative	Rice	ACTGCA	SRS715482	10001	6551	2092	0.83	3694	483
	RWVF9	Vegetative	Rice	AGAGTC	SRS715483	7818	4985	1749	0.81	3110	575
	RWRF3	Reproductive	Rice	ATCGAT	SRS715487	10831	7284	2301	0.84	4076	606
	RWRF6	Reproductive	Rice	ATGCTA	SRS715488	13418	7135	2055	0.85	3636	503
	RWRF9	Reproductive	Rice	CACAGT	SRS715489	4950	3111	1328	0.74	2739	532
	RWMF3	Maturity	Rice	CGCGCG	SRS715493	8464	5920	1613	0.86	2878	338
16S rDNA	RWMF6	Maturity	Rice	CGTATA	SRS715494	4079	2815	1255	0.73	2506	546
	RWMF9	Maturity	Rice	GACTAG	SRS715495	6081	3225	1339	0.75	2836	566
	MMRF4	Repoductive	Maize	CAGTCA	SRS715490	6108	3290	1269	0.78	2575	454
	MMRF7	Reproductive	Maize	CATGAC	SRS715491	5609	3112	1244	0.77	2321	473
	MMRF10	Reproductive	Maize	CGATAT	SRS715492	5449	3689	1331	0.80	2494	345
	MMVF4	-	Unplanted	AGCTGA	SRS715484	11301	7849	2233	0.86	3850	521
	MMVF7	-	Unplanted	AGTCAG	SRS715485	5719	3102	1251	0.77	2419	520
	MMVF10	-	Unplanted	ATATCG	SRS715486	7679	4264	1470	0.81	2572	442
	RWVF3	Vegetative	Rice	ACGTAC	SRS715481	12186	10275	2332	0.91	3501	511
	RWVF6	Vegetative	Rice	ACTGCA	SRS715482	8936	6732	1952	0.86	3141	533
	RWVF9	Vegetative	Rice	AGAGTC	SRS715483	9028	6831	1939	0.86	3302	464
	RWRF3	Reproductive	Rice	ATCGAT	SRS715487	10353	7672	2166	0.86	3606	586
	RWRF6	Reproductive	Rice	ATGCTA	SRS715488	12335	7107	1990	0.87	3230	459
	RWRF9	Reproductive	Rice	CACAGT	SRS715489	6965	4713	1560	0.84	2505	510
	RWMF3	Maturity	Rice	CGCGCG	SRS715493	5812	4344	1374	0.84	2371	304
16S rRNA	RWMF6	Maturity	Rice	CGTATA	SRS715494	3070	2062	980	0.71	1983	467
	RWMF9	Maturity	Rice	GACTAG	SRS715495	5440	3129	1249	0.78	2274	387
	MMRF4	Repoductive	Maize	CAGTCA	SRS715490	10351	5974	1675	0.86	2784	223
	MMRF7	Reproductive	Maize	CATGAC	SRS715491	7890	4481	1517	0.83	2520	229
	MMRF10	Reproductive	Maize	CGATAT	SRS715492	6226	4438	1422	0.85	2225	304
	MMVF4	-	Unplanted	AGCTGA	SRS715484	7452	6039	1790	0.86	2918	455
	MMVF7	-	Unplanted	AGTCAG	SRS715485	9622	6399	1781	0.87	2890	296
	MMVF10	-	Unplanted	ATATCG	SRS715486	10313	6230	1755	0.87	2859	466

*: number of sequences after quality analysis. Partial 16S rRNA primers: Bacteria: F515 (5'-GTGCCAGCNGCCGCGGTAA), R806 (5'-GGACTCVSGGGTATCTAAT). Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC)

Table 3.3: Number of archaeal sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047229 for archaeal sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Gene	*Name*	*Growth Stage*	*Plant*	*Barcode*	*Accession*	*Raw seqs*	*No. seqs**	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*
	RWVF3	Vegetative	Rice	ACGTAC	SRS715481	4063	1956	164	0.97	208	32
	RWVF6	Vegetative	Rice	ACTGCA	SRS715482	2572	1326	140	0.96	205	24
	RWVF9	Vegetative	Rice	AGAGTC	SRS715483	4886	2828	177	0.98	293	29
	RWRF3	Reproductive	Rice	ATCGAT	SRS715487	7437	3753	231	0.98	359	30
	RWRF6	Reproductive	Rice	ATGCTA	SRS715488	5458	2410	188	0.97	264	32
	RWRF9	Reproductive	Rice	CACAGT	SRS715489	1414	929	112	0.95	168	18
	RWMF3	Maturity	Rice	CAGTCA	SRS715493	6656	3630	202	0.98	291	25
16S rDNA	RWMF6	Maturity	Rice	CATGAC	SRS715494	4312	2137	176	0.97	284	31
	RWMF9	Maturity	Rice	CGATAT	SRS715495	6515	3250	201	0.97	492	26
	MMRF4	Repoductive	Maize	CAGTCA	SRS715490	8727	4644	206	0.99	256	17
	MMRF7	Reproductive	Maize	CATGAC	SRS715491	7723	3378	192	0.98	251	29
	MMRF10	Reproductive	Maize	CGATAT	SRS715492	6280	2894	164	0.99	194	23
	MMVF4	-	Unplanted	AGCTGA	SRS715484	6678	3369	168	0.99	215	10
	MMVF7	-	Unplanted	AGTCAG	SRS715485	2918	1504	128	0.97	185	16
	MMVF10	-	Unplanted	ATATCG	SRS715486	3909	2049	159	0.98	208	23
	RWRF3	Reproductive	Rice	ATCGAT	SRS715487	3232	1962	169	0.97	232	36
	RWRF6	Reproductive	Rice	ATGCTA	SRS715488	2797	1727	128	0.97	239	25
	RWRF9	Reproductive	Rice	CACAGT	SRS715489	572	565	74	0.95	126	19
16S rRNA	MMRF4	Repoductive	Maize	CAGTCA	SRS715490	7914	804	74	0.96	115	8
	MMRF7	Reproductive	Maize	CATGAC	SRS715491	1459	775	85	0.96	109	14
	MMRF10	Reproductive	Maize	CGATAT	SRS715492	831	532	55	0.96	78	10

*: number of sequences after quality analysis.
Partial 16S rRNA primers:
Archaea: Arch344F (5'-ACGGGGYGCAGCAGGCGCGA), Arch934br (5'-GTGCTCCCCCGCCAATTCCT)
Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC)

The sequences of ribosomal RNA were composed of the same archaeal lineages as the ribosomal gene-based community, but exhibited different relative abundances (Supplement Figure 3.3B). The most dominant class was *Methanomicrobia* (38-61% relative abundance) followed by Soil Crenarchaeotic Group (18-31%), Misc. Crenarchaeotic Group (4%) and *Methanobacteria* (2-6%). The archaeal community composition was not significantly different between fields cultivated with rice and maize on class, order and family level (ADONIS, P> 0.005). Nevertheless, specific archaeal groups changed in abundance. A significant increase of Soil Crenarchaeotic Group in the maize fields was observed (Supplement Figure 3.3B). Within Soil Crenarchaeotic Group Candidatus *Nitrososphaera* was higher in maize cultivated fields (data not shown). Only the top 30 OTUs representing up to 80% of all sequences were used for analysis (Figure 3.7). Again, there was a trend that methanogenic archaeal lineages (*Methanosarcina*, *Methanosaeta*, *Methanocella*, *Methanobacterium*) were more abundant in the rice than in the maize cultivated soil, and that non methanogenic groups (Soil Crenarchaeotic Group, GOM Arc I, Misc. Crenarchaeotic Group, Candidatus *Nitrososphaera*) were in particular observed in the maize field, but the trend was not statistically significant.

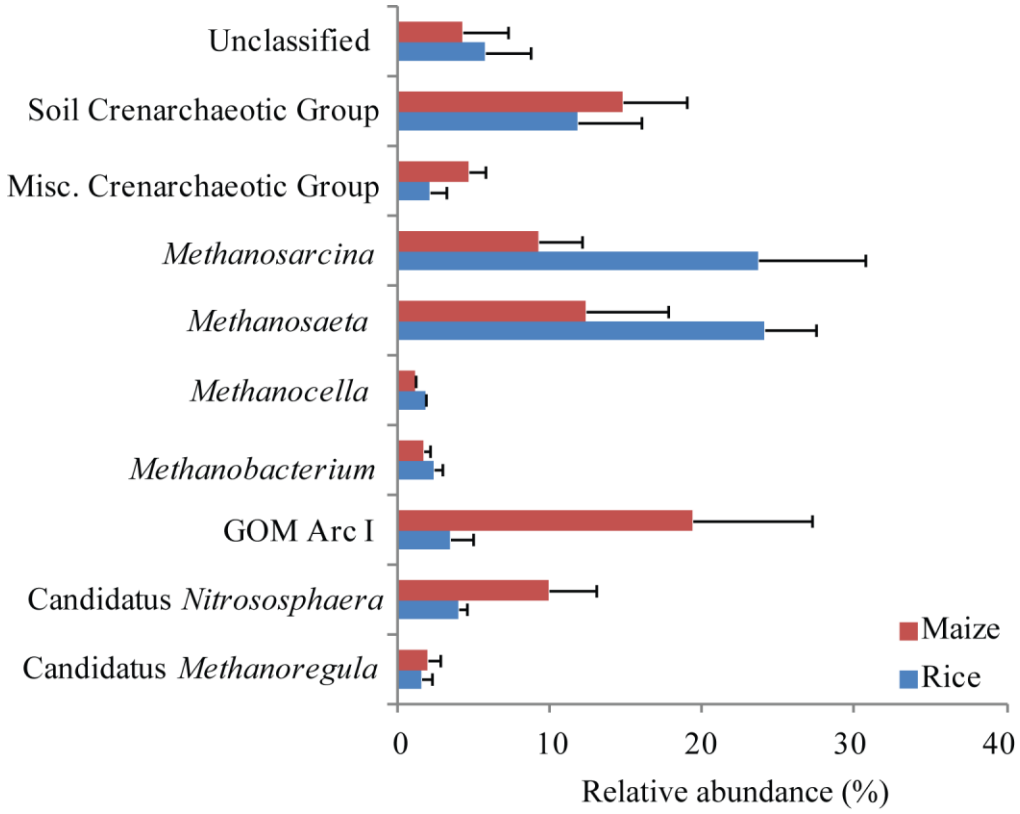


Figure 3.7 Relative abundance of the archaeal OTUs in rice field soil. Based on relative abundance top 30 OTUs derived from pyrosequencing of the archaeal 16S rRNA were grouped according to their phylogenetic assignment. OTUs were monitored in rice (blue) and maize (red) fields. Columns represent mean and bars standard errors of n=3.

3.5 Discussion

3.5.1 Bacterial and archaeal communities at different rice growth stages

Total bacterial and archaeal 16S rDNA copy numbers increased during reproductive growth stage indicating growth. Recently, Lee *et al.* (2014) likewise described in a Korean rice field an increase in bacterial and archaeal copy numbers during rice plant growth followed by a decrease at plant maturity. Although the microbial numbers in our Philippine soil were one order of magnitude higher than in the Korean soil (Lee *et al*., 2014), in both soils the microbial numbers changed by only a factor of two over the season. A similar observation was made by Itoh *et al*. (2013) showing a moderate increase of the resident microbes during rice plant growth under flooded conditions in a Japanese rice field. Textbook knowledge tells that plants secrete a complex mixture of organic and inorganic compounds (rhizodeposits) via their root system. Several studies showed an increase in root exudation with rice plant growth reaching a maximum at reproductive stage and decreasing again towards maturity of the rice plants (Aulakh *et al*., 2001; Lu *et al*., 2002; Watanabe *et al*., 2004; Pump and Conrad, 2014). Therefore, it is likely that the modest increase from vegetative to reproductive and the decrease towards maturity of the bacterial and archaeal abundance is driven by root exudation.

In contrast to the change in the resident (16S rDNA) populations, the numbers of the active populations (16S rRNA) were stable and did not show seasonal dynamics. Studies comprehensively covering the resident and active archaeal as well as bacterial communities in rice fields during plant growth are hardly available (Itoh *et al*., 2013). The detection of ribosomal RNA is equivalent to that of ribosomes, which are indicative for actively dividing or actively metabolizing microbial cells (Blazewicz *et al*., 2013). Therefore, changes in the 16S rRNA pool caused by growing cells can be superimposed by fluctuations in the amount of active but non-growing cells. Additionally, it was shown that different taxa can have different numbers of 16S rDNA copies (e.g. Sukenik *et al*., 2012). Interestingly, the standard errors of the abundance of both active bacterial and archaeal populations were higher in the vegetative and reproductive plant growth phase and decreased with plant maturity. This may be an indication for the influence of root exudation affecting microbial activity. All together the data indicates that the bacterial and archaeal communities were composed of active and

growing cells being enhanced during the reproductive growth stage possibly due to root exudation.

The composition of both the resident (16S rDNA) and the active (16S rRNA) bacterial community revealed only minor changes with the rice plant growth stages. Among the resident bacteria, only OTUs with negligible relative abundance were found to be specific for a particular plant growth stage. E.g., an OTU identified as *Anaeromyxobacter* was specific for the vegetative growth stage. *Anaeromyxobacter* spp. are known as iron reducers and are possibly important for carbon and iron dynamics in the rice rhizosphere (Ratering and Schnell, 2001; Treude *et al*., 2003). In rice fields oxidants like ferric iron are rapidly reduced (Conrad *et al*., 2014), but may be regenerated when plants allow O_2 release from their roots, thus as during vegetative plant growth (Liesack *et al*., 2000). Among the active bacteria, *Cyanobacteria* were highest during the early vegetative growth phase possibly caused by the previous field preparation (i.e. puddling) mixing sun-exposed soil parts into the bulk. The increase in relative abundance of active *Verrucomicrobia* and *Bacteroidia* during reproductive plant growth may be a consequence of their ecophysiology, which is playing a role in carbon degradation (Sugano *et al*., 2005; Tanahashi *et al*., 2005; Kikuchi *et al*., 2007; Rui *et al*., 2009). A member of *Verrucomicrobia, i.e., Opitutus terrae,* was isolated from a paddy rice field as potential polysaccharolytic and saccharolytic and capable of hydrogen production (Chin *et al*., 1999; 2001). *Bacteroidia* are known key players in decomposition of rice plant residue (Weber *et al*., 2001; Akasaka *et al*., 2003) and *Bacteroidetes* prominent heterotrophs in rice field soil including a propionate-producing fermentative representative (Akasaka *et al*., 2003). The OTU based analysis showed that the methanotrophic *Methylocystis* became prominent before plant maturity. *Methylocystis*, a type-II methanotroph, has commonly been found in rice field soil (Murase and Frenzel, 2007; Shrestha *et al*., 2010). Methanotrophs are dependent on their primary substrate methane and oxygen. Oxygen was probably released by the roots during the reproductive growth phase of the rice plant. For example Gilbert and Frenzel (1998) reported radial oxygen loss by roots of up to 6 weeks old rice plants.

The T-RFLP analysis in the Philippine rice fields showed a relatively constant composition of the archaeal community over the season. Similar results had been obtained in

an Italian rice field (Krüger *et al*., 2005). Our pyrosequencing data indicated an increase in relative abundance of the dominating methanogens (*Methanosarcinaceae*, *Methanosaetaceae*, *Methanobacteriaceae* and *Methanocellaceae*) during reproductive growth stage, but this increase was statistically not significant. Within the order of *Methanosarcinales* GOM Arc I species were notably detected under all tested conditions and decreased during reproductive stage. GOM Arc I was formerly known as ANME-2d caused by phylogenetic relation to the anaerobic methanotrophs ANME-2 (Mills *et al*., 2005; Martinez *et al*., 2006). Nevertheless, the role of GOM Arc I in the methane biogeochemistry is still unclear (Lloyd *et al*., 2006; Knittel and Boetius, 2009). We speculate that the importance of these organisms which were previously detected in relatively high numbers in South Korean rice field soil (Ahn *et al*., 2014) has been underestimated and strengthen the need to identify their function in methane cycling.

All together rhizodeposition and oxygen release seemed to increase growth and activity of specific bacterial and archaeal lineages during the reproductive growth stage. However, changes over the season were only small and the resident and active microbial communities remained relatively conserved.

3.5.2 Bacterial and archaeal communities in flooded and non-flooded fields

Rotation of the cultivated crop from paddy rice (flooded) to upland maize (non-flooded) changes the field conditions dramatically. In our study, we were dealing with flooded rice fields and non-flooded unplanted fields, which were then planted with maize, but kept under non-flooded conditions. Anaerobic degradation of organic matter to CH_4 is only possible if the bulk of the soil is anoxic, such as in flooded fields. In non-flooded fields no or comparatively little anaerobic microbial activity is expected. Indeed, compared to the flooded fields CH_4 emission was only minor from the non-flooded fields (Weller *et al*., 2014). Therefore, living conditions of obligately anaerobic microorganisms, such as methanogenic archaea and many fermenting bacteria, were restricted.

The abundances of resident bacteria and archaea (16S rDNA) were lowest in unplanted fields, whereas they were highest in the flooded rice fields and intermediate in the non-flooded maize fields. The microbial populations apparently increased in number when

the non-flooded fields were planted with maize, but did not reach the same level as in the flooded rice fields. Hence, microbial abundance was apparently affected by both flooding and the presence of vegetation. The low microbial abundance in unplanted fields was possibly due to the absence of release of organic material from roots and/or lack of fertilization, which allowed the microbes to grow to some extent in the maize fields. In these oxic soils, however, the number of microbes remained lower than in the anoxic flooded fields. Surprisingly, the abundance of ribosomal RNA, being indicative for active microbes, was in the same range for both unplanted and planted fields and for both non-flooded maize and flooded rice fields. Therefore, we assume that the microbial cells in non-flooded unplanted soil, and to some extend also the maize field soil, contained more ribosomal RNA than those in the flooded rice field soil. The rather high ratio of rRNA/rDNA in non-flooded fields was observed in most replicate samples, but there were a few replicates, which behaved differently. High ratios of rRNA/rDNA have also been observed in non-flooded Japanese rice fields but not been further discussed (Watanabe *et al*., 2007). However, numbers of rRNA decreasing with drainage have also been observed in a Japanese rice field (Itoh *et al*., 2013). At a first glance high ratios of rRNA/rDNA seem surprising, since anaerobic microorganisms should be less active in the unplanted and maize fields than in the flooded rice fields. However, it has been shown that even dormant cells harbor measureable amounts of 16S rRNA and that in some cases the 16S rRNA amount can even be significantly higher than in vegetative cells (Chambon *et al*., 1968; Sukenik *et al*., 2012). The maintenance of a high level of ribosomal RNA under unfavorable conditions is interpreted as preparedness for activity when conditions improve. Hence, we assume that numbers of anaerobic microorganisms decreased when the flooded rice fields were turned into non-flooded maize fields, but at the same time increased the cellular levels of rRNA (presumably ribosomes) as a stress response and possibly to be prepared for new flooding.

However, there may be additional explanations for the high ratio of rRNA/rDNA in the non-flooded soil. For example, during field preparation and after drainage soil structure gets disturbed, and this process may cause death of microbes by breaking up the cells. The nutrients of the dead cells become then available for the surviving microbes and may thus enhance their activity. The soil texture was a silt loam, which is characterized by a high water holding capacity. The high water holding capacity may have allowed the maintenance

of anaerobic microniches with active populations of anaerobic microorganisms. Finally, drainage may have allowed an increase of the soil temperature, thus promoting the activity of the overall community which inhabits the anaerobic microniches. Interestingly, the microbial community compositions were not much different between flooded and non-flooded fields. Although CCA analysis of the community based on T-RFLP revealed some differences in composition, the variance on the two CCA axes were less than 16%. Only the analysis by 454 pyrosequencing unveiled some changes in relative abundance of few bacterial groups but no dramatic community shifts. In the present study these bacterial lineages can be grouped due to their ecophysiology. *Spartobacteria* and *Sphingobacteria* were both described as aerobes and increased in their relative abundance in the non-flooded fields possibly due to decreasing water level and concomitant increased oxygen exposure. The first isolate of *Spartobacteria* was described as an aerobic heterotrophic bacterium able to grow on saccharide components of plant biomass (Sangwan *et al*., 2004). Additionally, some members of the *Sphingobacteria* were described as aerobes, while others are anaerobes or facultative anaerobes suggesting a dependence on oxygen levels (Janssen, 2006). The second group of bacterial lineages (*Bacteroidetes* and *Acidobacteria)* increasing in non-flooded fields is associated with their ability to sustain low substrate conditions and to degrade complex organic compounds under anaerobic conditions. For instance *Bacteroidetes* were frequently detected during rice plant residue decomposition (e.g. Weber *et al*., 2001; Rui *et al*., 2009) and have the ability to grow on various complex carbon substrates (Kirchman, 2002). Although *Acidobacteria* are widely distributed and highly abundant in soil environments little is known about their ecology (Lee and Cho, 2009). Various observations suggest that the chemo-organotrophic and oligotrophic *Acidobacteria* are adapted to low substrate availability highlighted by slow growth rates (e.g. Davis *et al*., 2005, 2011) and are able to decompose complex carbon compounds like xylan, cellulose and pectin (Eichorst *et al*., 2011). The last bacterial lineage more pronounced in the non-flooded fields was *Myxococcales*. Iron reducing *Anaeromyxobacter* are members of *Myxococcales* and represented the maturity of the order in the present study. During drainage regeneration of inorganic electron acceptors like ferric iron occurs. Therefore, it is likely that iron reducers out of *Myxococcales* are supported in their competition with methanogens for electron acceptors like hydrogen and acetate (Ratering and Conrad, 1998). In summary, the

bacterial groups in the unplanted fields were characterized by their abilities to grow under oxic conditions and to degrade complex carbon substrates.

Similarly, the archaeal community composition was quite similar in non-flooded and flooded fields. This observation is consistent with previous studies showing that crop rotation including upland crop management affected archaeal communities only little (Watanabe *et al*., 2006; 2011; Fernandez Scavino *et al*., 2013). However, we observed a significant increase in relative abundance of *Methanosarcinaceae* in upland maize fields by T-RFLP analysis and a non-significant increase by pyrosequencing. In Japanese rice fields *Methanosarcinales* were a major group under both flooded and drainage conditions (Watanabe *et al*., 2009; Itoh *et al*., 2013). *Methanosarcina* spp. together with *Methanocella* spp. have also been found in dry ecosystems, such as upland soils and desert biological soil crusts (Nicol *et al*., 2003; Angel *et al*., 2012; Conrad *et al*., 2012; Aschenbach *et al*., 2013). These species possess a relatively large number of genes coding for oxygen-detoxifying enzymes (Erkel *et al*., 2006), thus probably allowing them to survive exposition to oxygen in dry soils (Angel *et al*., 2011; Angel *et al*., 2012). Therefore, it is likely that *Methanosarcina* spp. survived relatively well when the flooded rice fields were turned into non-flooded maize fields, thus increasing their relative abundance among the other archaea.

The Soil Crenarchaeotic Group also showed a relatively high abundance in the non-flooded maize fields. The ecophysiology of *Crenarchaeota* is largely unknown (Pester *et al*., 2011), although *Thaumarchaeota*, with potential for ammonia oxidation are found as a dominant archaeal group in aerated soils (e.g. Nicol *et al*., 2003). An upland pasture in Uruguay was reported to be dominated by *Crenarchaeota*/*Thaumarchaeota*, which decreased in relative abundance as soon as the soil was turned into a pasture-rice crop rotation (Fernandez Scavino *et al*., 2013). The predominance of *Methanosarcinaceae* and Soil Crenarchaeotic Group in non-flooded soils emphasizes their capability to withstand temporal desiccation and oxygen stress.

3.5.3 Conclusion

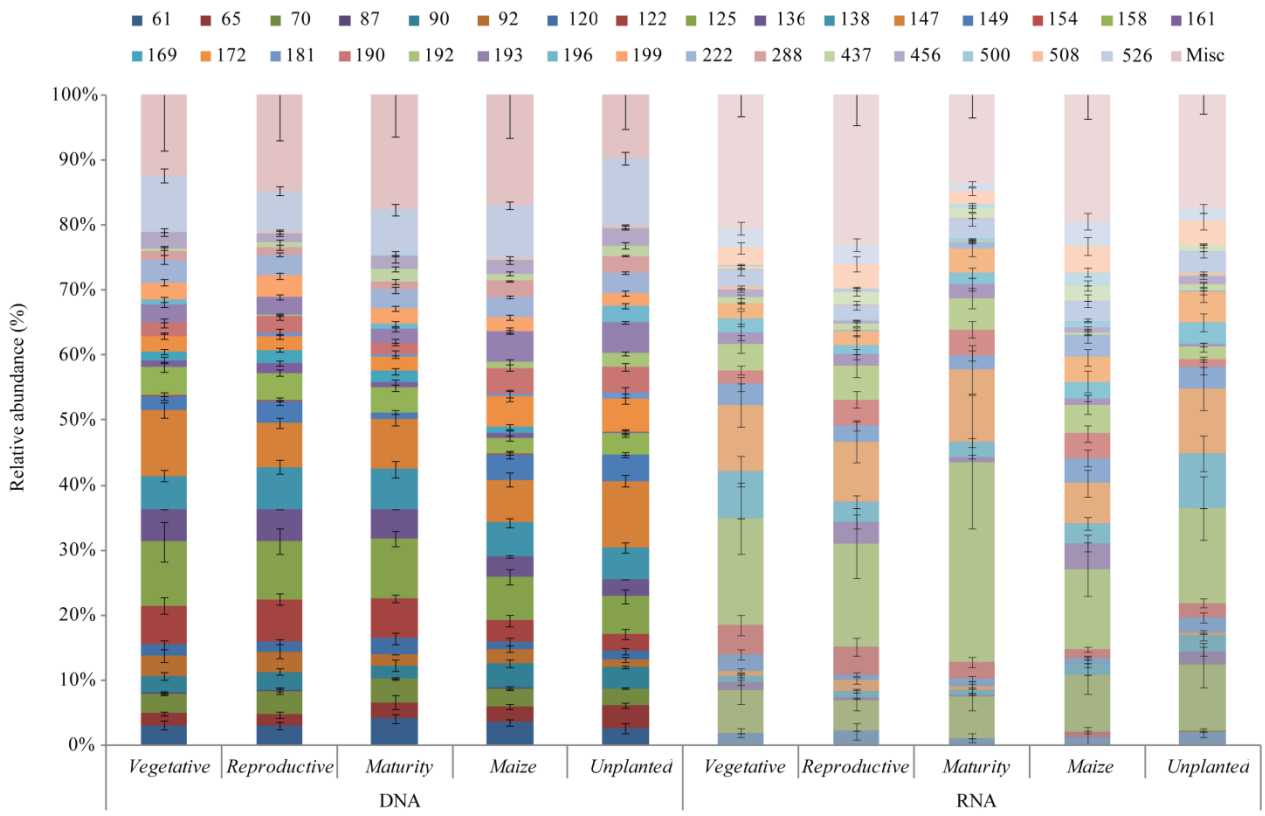
The bacterial and archaeal abundance and activity only moderately changed during rice growth most likely by the influence of rice plants and its root exudation. However, neither archaeal nor bacterial community composition changed much suggesting good adaptation to the conditions in the rice field. By contrast, the change from flooded rice to non-flooded cropping caused a comparatively stronger change in the microbial community composition, which however, was also not very dramatic. The relatively minor effect of change to non-flooded cropping was probably caused by the fact that the microbial communities in the rice field soil were historically adapted to regular drainage. This adaptation was also seen by the maintenance of a high ratio of ribosomal RNA per gene copy, being equivalent to a high number of ribosomes per cell, indicating a preparedness for change between unfavourable non-flooded to favourable flooded conditions for the methanogenic archaea and anaerobic bacteria resident in the rice field soil. The similarity in composition together with the statistically significant increase in ribosomal numbers imply that it was not so much specific members of the communities that regulated their ratios of rRNA/rDNA, but the communities in general that reacted upon the change from flooded to non-flooded state. We conclude that methods reducing greenhouse gas emission from rice fields like mid-season drainage and crop rotation (Wassmann *et al*., 2000; Li *et al*., 2006; Pittelkow *et al*., 2014) will have only little immediate effect on the bacterial and archaeal communities and thus, allow their function to be largely conserved over unfavourable periods.

3.6 Supplemental material

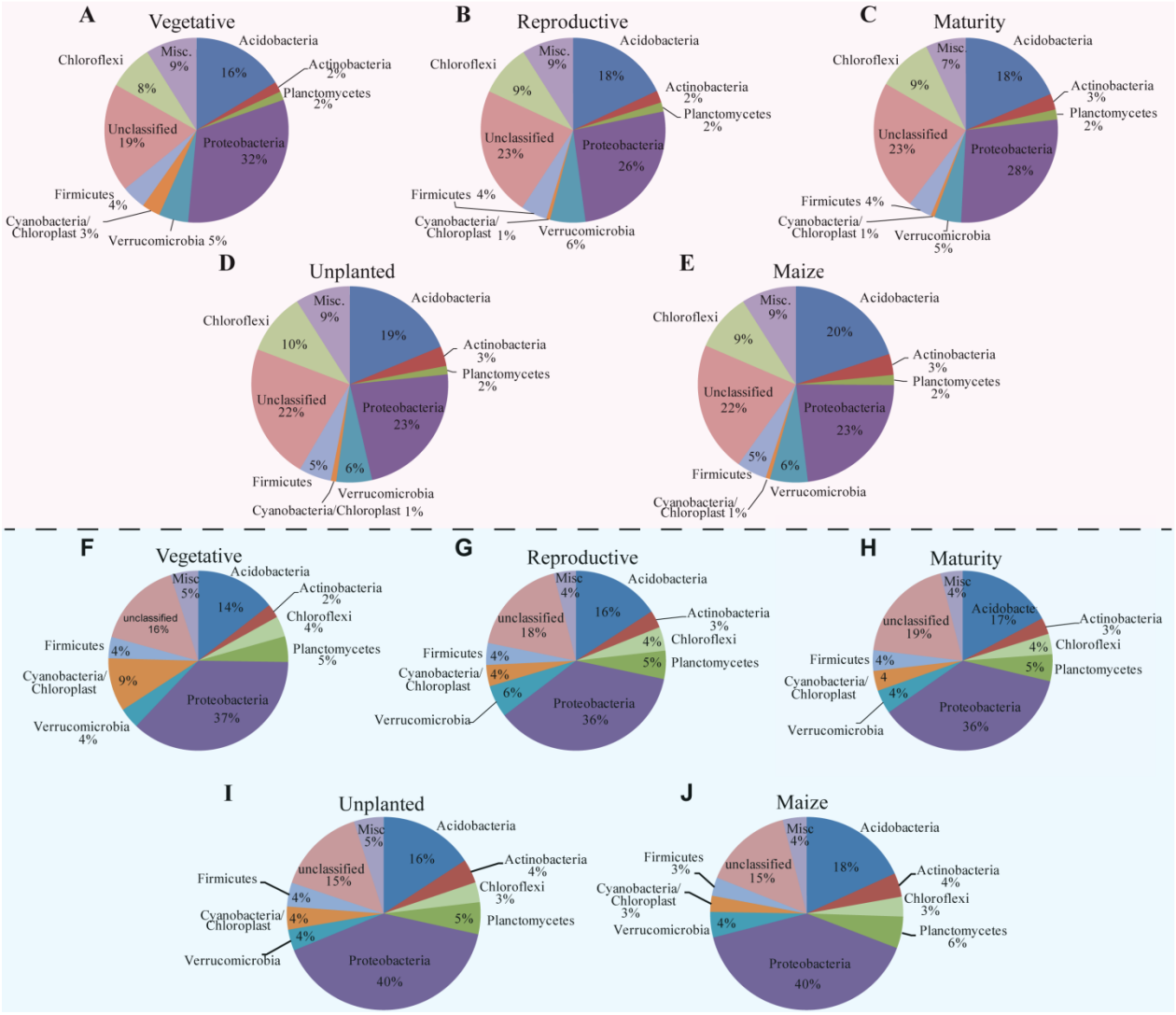
Supplement Table 3.1: Proportion of variance explained (percentage of total variation) by environmental variables determined by CCA for the resident (16s rDNA) and active (16S rRNA) bacterial and archaeal community based on T-RFLP.

Community	***Variable***	***% Variance explained***	***P-value***
16S rDNA Bacteria	Field management	12.0	0.01*
	Growth stage	10.5	0.01*
	Gravimetric water content	5.8	0.01*
16S rRNA Bacteria	Field management	14.4	0.01*
	Growth stage	21.4	0.01*
	Gravimetric water content	5.7	0.01*
16S rDNA Archaea	Field management	16.2	0.01*
	Growth stage	22.8	0.01*
	Gravimetric water content	7.5	0.02*
16S rRNA Archaea	Field management	15.7	0.01*
	Growth stage	11.2	0.01*
	Gravimetric water content	11.7	0.01*

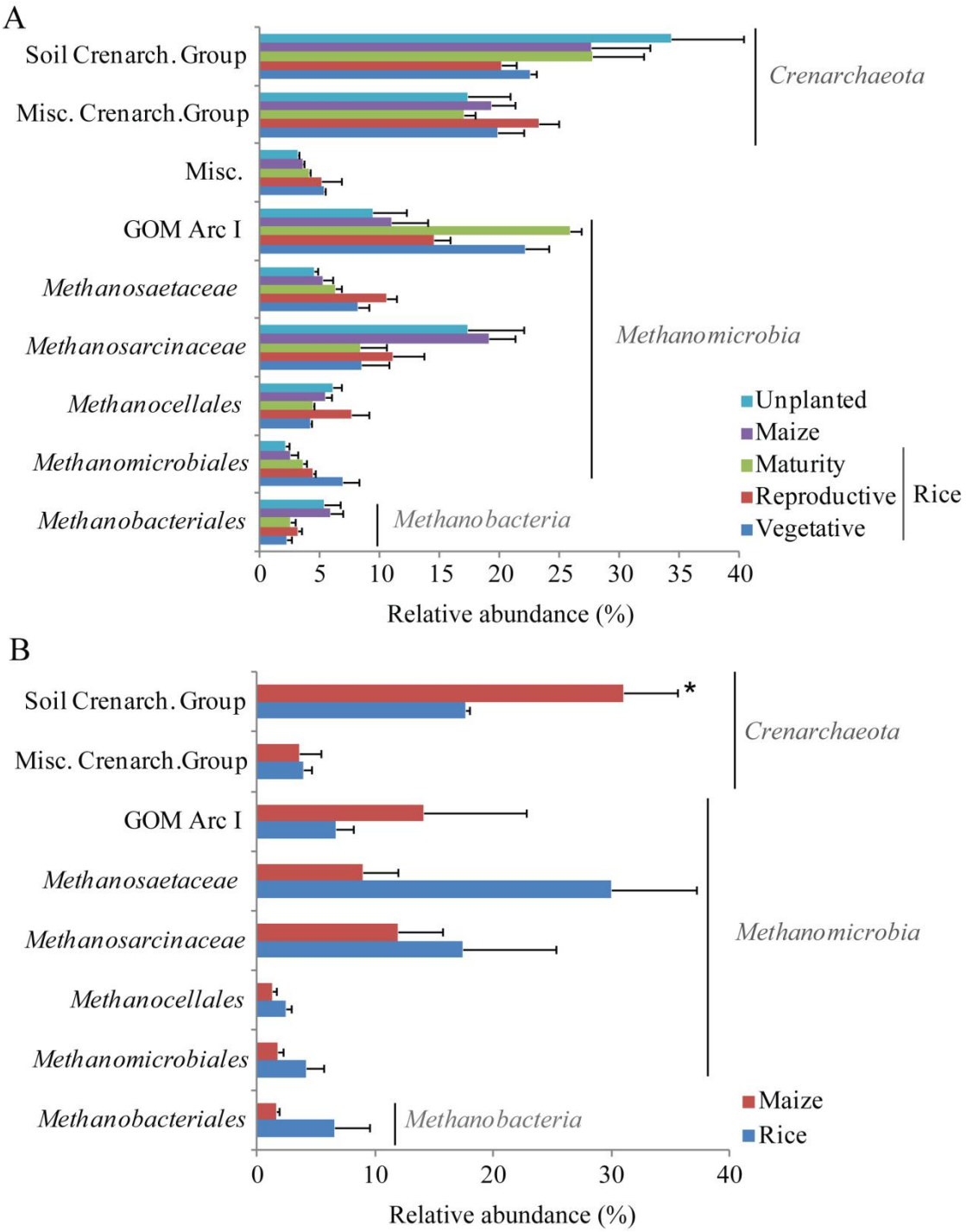
*: significant. Significance was tested by ANOVA.



Supplement Figure 3.1 Histograms of the relative abundance of T-RFs obtained from T-RFLP analysis of bacterial 16S rDNA (left, dark columns) and rRNA (right, light columns) during rice plant growth and in non-flooded fields (unplanted, maize). Bacterial T-RFs with minimum 2% of relative abundance in at least one sample are mapped. Remaining T-RFs were summarized as Misc. Bars represent standard errors of n=9.



Supplement Figure 3.2 Relative abundance of the dominant bacterial phyla detected in rice field soil by pyrosequencing of 16S rDNA/rRNA. Bacterial phyla based on 16S rDNA during different rice plant growth stages **(A-C)** as well as in unplanted **(D)** and maize cultivated fields **(E)** and bacterial phyla based on 16S rRNA during different rice plant growth stages **(F-H)** as well as in unplanted **(I)** and maize cultivated fields **(J)** are displayed. Only phyla with a minimum of 2% relative abundance are shown, those with <2% are summarized as Misc. (n=3).



Supplement Figure 3.3 Relative abundance of the archaeal lineages detected in rice field soil by pyrosequencing of 16S rDNA **(A)** and of 16S rRNA **(B)**. Archaeal lineages with minimum 2% of relative abundance in at least one sample are mapped. Remaining phyla were summarized as Misc. Columns represent mean and bars standard errors of n=3. Asterisk indicates significant difference.

3.7 Acknowledgement

This work has been funded as part of the ICON consortium (BR2238/9-1). We are thankful to Martin B. Blaser for his organizational work, support during sample collection and valuable comments on the study design. We thank Peter Frenzel for valuable comments on the study design and his support during data analysis. Furthermore, we thank the International Rice Research Institute and Reiner Wassmann for providing research space and support during sample collection. We thank Mary Louise Mendoza, Eugene Aquino and Jerico Stefan Bigornia for sample collection. We are thankful to Franziska B. Brandt for valuable comments on the manuscript. We thank MPGC in Cologne for access to the sequencing facility and support.

3.8 References

Ahn, J. H., Choi, M. Y., Kim, B. Y., Lee, J. S., Song, J., Kim, G. Y., and Weon, H. Y. (2014). Effects of water-saving irrigation on emissions of greenhouse gases and prokaryotic communities in rice paddy soil. *Microb. Ecol.* 68, 271-283. doi: 10.1007/s00248-014-0371-z

Angel, R., Matthies, D., and Conrad, R. (2011). Activation of methanogenesis in arid biological soil crusts despite the presence of oxygen. *PLoS ONE* 5:e20453. doi:10.1371/journal.pone.0020453

Angel, R., Claus, P., and Conrad, R. (2012). Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *ISME J.* 6, 847-862.

Asakawa, S., and Kimura, M. (2008). Comparison of bacterial community structures at main habitats in paddy field ecosystem based on DGGE analysis. *Soil. Biol. Biochem.* 40, 1322-1329.

Akasaka, H., Izawa, T., Ueki, K., and Ueki, A. (2003). Phylogeny of numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil. *FEMS Microbiol. Ecol.* 43, 149-161.

Aschenbach, K., Conrad, R., Řeháková, K., Doležal, J., Janatková, K., and Angel, R. (2013). Methanogens at the top of the world: occurrence and potential activity of methanogens in newly deglaciated soils in high-altitude cold deserts in the Western Himalayas. *Front. Microbio*. 4:359. doi:10.3389/fmicb.2013.00359

Aulakh, M. S., Wassmann, R., Bueno, C., Kreuzwieser, J., and Rennenberg, H. (2001). Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biol*. 3, 139-148.

Bates, S. T., Cropsey, G. W., Caporaso, J. G., Knight, R., and Fierer, N. (2011). Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol*. 77, 1309-1314.

Blazewicz, S. J., Barnard, R. L., Daly, R. A., and Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. *ISME J*. 7, 2061-2068.

Bridgham, S. D., Cadillo-Quiroz, H., Keller, J. K., and Zhuang, Q. (2013). Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. *Glob. Change Biol*. 19, 1325-1346.

Bürgmann, H., Pesaro, M., Widmer, F., and Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. *J. Microbiol. Meth*. 45, 7-20.

Burggraf, S., Huber, H., Stetter, K.O. (1997). Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *Int. J. Syst. Evol. Microbiol*. 47, 657-660.

Casamayor, E. O., Massana, R., Benlloch, S., Øvreås, L., Díez, B., Goddard, V. J., *et al*. (2002). Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. *Environ. Microbiol*. 4, 338-348.

Chambon, P., DuPraw, E.J., and Kornberg, A. (1968). Biochemical studies of bacterial sporulation and germination. *J. Biol. Chem*. 243, 5101-5109.

Chen, Y. H., and Prinn, R. G. (2005). Atmospheric modeling of high-and low-frequency methane observations: Importance of interannually varying transport. *J. Geophys. Res*. 110, doi: 10.1029/2004JD005542

Chin, K.-J., Hahn, D., Hengstmann, U., Liesack, W., and Janssen, P.H. (1999). Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. *Appl. Environ. Microbiol.* 65, 5042-5049.

Chin, K. J., Liesack, W., and Janssen, P. H. (2001). *Opitutus terrae* gen. nov., sp. nov., to accommodate novel strains of the division '*Verrucomicrobia*' isolated from rice paddy soil. *Int. J. Syst. Evol. Micr*. 51, 1965-1968.

Conrad, R. (2007). Microbial ecology of methanogens and methanotrophs. *Adv. Agron.* 96,1-63.

Conrad, R. (2009). The global methane cycle: recent advances in understanding the microbial processes involved. *Environ. Microbiol. Rep*. 1, 285-292.

Conrad, R., Claus, P., Chidthaisong, A., Lu, Y., Fernandez Scavino, A., Liu, Y., *et al*. (2014). Stable carbon isotope biogeochemistry of propionate and acetate in methanogenic soils and lake sediments. *Org. Geochem.* 73, 1-7.

Conrad, R., and Klose, M. (2006). Dynamics of the methanogenic archaeal community in anoxic rice soil upon addition of straw. *Eur. J. Soil. Sci.* 57, 476-484.

Conrad, R., Klose, M., Lu, Y., and Chidthaisong, A. (2012). Methanogenic pathway and archaeal communities in three different anoxic soils amended with rice straw and maize straw. *Front. Microbiol.* 3:4. doi:10.3389/fmicb.2012.00004

Conrad, R., Klose, M., and Noll, M. (2009). Functional and structural response of the methanogenic microbial community in rice field soil to temperature change. *Environ. Microbiol.* 11, 1844-1853.

Conrad, R., Klose, M., Noll, M., Kemnitz, D., and Bodelier, P.L.E. (2008). Soil type links microbial colonization of rice roots to methane emission. *Glob. Change Biol.* 14, 657-669.

Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G., and Smalla, K. (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiol. Ecol.* 56, 236-249.

Davis, K. E., Joseph, S. J., and Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl. Environ. Microbiol.* 71, 826-834.

Davis, K. E., Sangwan, P., and Janssen, P. H. (2011). *Acidobacteria, Rubrobacteridae* and *Chloroflexi* are abundant among very slow-growing and mini-colony-forming soil bacteria. *Environ. Microbiol*. 13, 798-805.

Dennis, P. G., Miller, A. J., and Hirsch, P. R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities?. *FEMS Microbiol. Ecol.* 72, 313-327.

Dunbar, J., Ticknor, L.O., and Kuske, C.R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol*. 67, 190-197.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996-998.

Eichorst, S. A., Kuske, C. R., and Schmidt, T. M. (2011). Influence of plant polymers on the distribution and cultivation of bacteria in the phylum *Acidobacteria*. *Appl. Environ. Microbiol.* 77, 586-596.

Erkel, C., Kube, M., Reinhardt, R., and Liesack, W. (2006). Genome of Rice Cluster I archaea—the key methane producers in the rice rhizosphere. *Science* 313, 370-372.

Fernandez Scavino, A., Ji, Y., Pump, J., Klose, M., Claus, P., and Conrad, R. (2013). Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated rice fields. *Environ. Microbiol*. 15, 2588-2602.

Forster, P., Ramaswamy, P., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W., *et al*. (2007). "Changes in atmospheric constituents and in radiative forcing," in *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M., and Miller, H. L., Cambridge University Press, Cambridge, United Kingdom, 129-234.

Gilbert, B., and Frenzel, P. (1998). Rice roots and CH_4 oxidation: the activity of bacteria, their distribution and the microenvironment. *Soil. Biol. Biochem.* 30, 1903-1916.

Großkopf, R., Janssen, P.H., and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64, 960-969.

Grayston, S. J., Wang, S., Campbell, C. D., and Edwards, A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil. Biol. Biochem.* 30, 369-378.

Heinz, E., Kraft, P., Buchen, C., Frede, H. G., Aquino, E., and Breuer, L. (2013). Set up of an automatic water quality sampling system in irrigation agriculture. *Sensors* 14, 212-228.

Itoh, H., Ishii, S., Shiratori, Y., Oshima, K., Otsuka, S., Hattori, M., and Senoo, K. (2013). Seasonal transition of active bacterial and archaeal communities in relation to water management in paddy soils. *Microbes Environ*. 28, 370-380.

Janssen, P. H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* 72, 1719-1728.

Kikuchi, H., Watanabe, T., Jia, Z., Kimura, M., and Asakawa, S. (2007). Molecular analyses reveal stability of bacterial communities in bulk soil of a Japanese paddy field: estimation by denaturing gradient gel electrophoresis of 16S rRNA genes amplified from DNA accompanied with RNA. *Soil Sci. Plant Nutr.* 53, 448-458.

Kimura, M., Murase, J., and Lu, Y. (2004). Carbon cycling in rice field ecosystems in the context of input, decomposition and translocation of organic materials and the fates of their end products (CO_2 and CH_4). *Soil. Biol. Biochem.* 36, 1399-1416.

Kirchman, D. L. (2002). The ecology of *Cytophaga–Flavobacteria* in aquatic environments. *FEMS Microbiol. Ecol.* 39, 91-100.

Knittel K., and Boetius, A. (2009). Anaerobic oxidation of methane: progress with an unknown process. *Annu. Rev. Microbiol.* 63, 311-334.

Kowalchuk, G. A., Yergeau, E., Leveau, J. H. J., Sessitsch, A., and Bailey, M. (2010). "Plant-associated microbial communities," in *Environmental molecular microbiology,* eds. Liu, W.-T., and Jansson, J. K., Caister Academic Press, Poole, United Kingdom, 131-148.

Krüger, M., Frenzel, P., Kemnitz, D., and Conrad, R. (2005). Activity, structure and dynamics of the methanogenic archaeal community in a flooded Italian rice field. *FEMS Microbiol. Ecol.* 51, 323-331.

Lee, S. H., and Cho, J. C. (2009). Distribution patterns of the members of phylum acidobacteria in global soil samples. *J. Microbiol. Biotech*. 19, 1281-1287.

Lee, H. J., Kim, S. Y., Kim, P. J., Madsen, E. L., and Jeon, C. O. (2014). Methane emission and dynamics of methanotrophic and methanogenic communities in a flooded rice field ecosystem. *FEMS Microbiol. Ecol.* 88, 195-212.

Li, C.S., Salas, W., DeAngelo, B., and Rose, S. (2006). Assessing alternatives for mitigating net greenhouse gas emissions and increasing yields from rice production in China over the next twenty years. *J. Environ. Qual.* 35, 1554-1565.

Liesack, W., Schnell, S., and Revsbech, N. P. (2000). Microbiology of flooded rice paddies. *FEMS Microbiol. Rev*. 24, 625-645.

Lloyd, K.G., Lapham, L., and Teske, A. (2006). An anaerobic methane-oxidizing community of ANME-1b archaea in hypersaline Gulf of Mexico sediments. *Appl. Environ. Microbiol.* 72, 7218-7230.

Lopes, A. R., Manaia, C. M., and Nunes, O. C. (2014). Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. *FEMS Microbiol. Ecol.* 87, 650-663.

Lu, Y., Watanabe, A., and Kimura, M. (2002). Contribution of plant-derived carbon to soil microbial biomass dynamics in a paddy rice microcosm. *Biol. Fert. Soils* 36, 136-142.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Buchner, A., *et al*. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res*. 32, 1363-1371.

Lueders, T., and Friedrich, M. W. (2003). Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl. Environ. Microbiol.* 69, 320-326.

Martinez, R.J., Mills, H.J., Story, S., and Sobecky, P.A. (2006). Prokaryotic diversity and metabolically active microbial populations in sediments from an active mud volcano in the Gulf of Mexico. *Environ. Microbiol.* 8, 1783-1796.

Marschner, P., Yang, C. H., Lieberei, R., and Crowley, D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil. Biol. Biochem.* 33, 1437-1445.

Mills, H. J., Martinez, R. J., Story, S., and Sobecky, P. A. (2005). Characterization of microbial community structure in Gulf of Mexico gas hydrates: comparative analysis of DNA- and RNA-derived clone libraries. *Appl. Environ. Microbiol.* 71, 3235-3247.

Murase, J., and Frenzel, P. (2007). A methane-driven microbial food web in a wetland rice soil. *Environ. Microbiol.* 9, 3025-3034.

Muyzer, G., Teske, A., Wirsen, C.O., Jannasch, H.W. (1995). Phylogenetic relationship of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* 164, 165-172.

Nicol, G. W., Glover, L. A., and Prosser, J. I. (2003). The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. *Environ. Microbiol.* 5, 152-162.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382-395.

Oksanen, J., Blanchet, G. F., Kindt, R., Legendre, R., Minchin, P. R., O'Hara, R. B., *et al*. (2012). vegan: Community Ecology Package ver. 2.0-5.

Osborne, C.A., Galic, M., Sangwan, P., and Janssen, P.H. (2005). PCR-generated artefact from 16S rRNA gene-specific primers. *FEMS Microbiol. Lett.* 248, 183-187.

Pester, M., Schleper, C., and Wagner, M. (2011). The *Thaumarchaeota*: an emerging view of their phylogeny and ecophysiology. *Curr. Opin. Microbiol.* 14, 300-306.

Peng, J. J., Lü, Z., Rui, J., and Lu, Y. H. (2008). Dynamics of the methanogenic archaeal community during plant residue decomposition in an anoxic rice field soil. *Appl. Environ. Microbiol.* 74, 2894-2901.

Pittelkow, C. M., Adviento-Borbe, M. A., Kessel, C., Hill, J. E., and Linquist, B. A. (2014). Optimizing rice yields while minimizing yield-scaled global warming potential. *Glob. Change Biol.* 20, 1382-1393.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W.,Peplies, J., and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188-7196.

Pump, J., and Conrad, R. (2014). Rice biomass production and carbon cycling in $^{13}CO_2$ pulse-labeled microcosms with different soils under submerged conditions. *Plant Soil* 384, 213-229.

Ramakrishnan, B., Lueders, T., Dunfield, P.F., Conrad, R., and Friedrich, M.W. (2001). Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production. *FEMS Microbiol. Ecol.* 37, 175-186.

Ratering, S., and Conrad, R. (1998). Effects of short-term drainage and aeration on the production of methane in submerged rice soil. *Glob. Change Biol*. 4, 397-407.

Ratering, S., and Schnell, S. (2001). Nitrate-dependent iron (II) oxidation in paddy soil. *Environ. Microbiol.* 3, 100-109.

R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, Available online at: http://www.R-project.org/.

Rui, J., Peng, J., and Lu, Y. (2009). Succession of bacterial populations during plant residue decomposition in rice field soil. *Appl. Environ. Microbiol.* 75, 4879-4886.

Sangwan, P., Chen, X., Hugenholtz, P., and Janssen, P. H. (2004). *Chthoniobacter flavus* gen. nov., sp. nov., the first pure-culture representative of subdivision two, *Spartobacteria* classis nov., of the phylum *Verrucomicrobia*. *Appl. Environ. Microbiol.* 70, 5875-5881.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., *et al*. (2009). Introducing mothur: open-source, platform-independent, community-supported

software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537-7541.

Schloss, P. D., Gevers, D., and Westcott, S. L. (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS ONE* 6: e27310. doi:10.1371/journal.pone.0027310

Shrestha, P. M., Kube, M., Reinhardt, R., and Liesack, W. (2009). Transcriptional activity of paddy soil bacterial communities. *Environ. Microbiol.* 11, 960-970.

Shrestha, P.M., Noll, M., and Liesack, W. (2007). Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. *Environ. Microbiol.* 9, 2464-2474.

Shrestha, M., Shrestha, P. M., Frenzel, P., and Conrad, R. (2010). Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. *The ISME J.* 4, 1545-1556.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., *et al*. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* 67, 4742-4751.

Stubner, S. (2002). Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen™ detection. *J. Microbiol. Meth*. 50, 155-164.

Sugano, A., Tsuchimoto, H., Tun, C. C., Asakawa, S., and Kimura, M. (2005). Succession and phylogenetic profile of eubacterial communities in rice straw incorporated into a rice field: estimation by PCR-DGGE analysis. *Soil Sci. Plant Nutr.* 51, 51-60.

Sukenik, A., Kaplan-Levy, R. N., Welch, J. M., Post, A. F. (2012). Massive multiplication of genome and ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalis-porum* (Cyanobacteria). *ISME J.* 6, 670-679.

Tanahashi, T., Murase, J., Matsuya, K., Hayashi, M., Kimura, M., and Asakawa, S. (2005). Bacterial communities responsible for the decomposition of rice straw compost in a Japanese

rice paddy field estimated by DGGE analysis of amplified 16S rDNA and 16S rRNA fragments. *Soil Sci. Plant Nutr.* 51, 351-360.

Treude, N., Rosencrantz, D., Liesack, W., and Schnell, S. (2003). Strain FAc12, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales*. *FEMS Microbiol. Ecol.* 44, 261-269.

Uren, N. C. (2007). "Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants," in *The rhizosphere*. *Biochemistry and organic substances at the soil-plant interface*, eds. Pinton, R., Varanini, Z., and Nannipieri, P., Marcel Dekker, New York, USA, 1-21.

Van Nguyen, N., and Ferrero, A. (2006). Meeting the challenges of global rice production. *Paddy Water Environ.* 4, 1-9.

Wassmann, R., Lantin, R. S., Neue, H. U., Buendia, L. V., Corton, T. M., and Lu, Y. (2000). Characterization of methane emissions from rice fields in Asia. III. Mitigation options and future research needs. *Nutr. Cycl. Agroecosys.* 58, 23-36.

Watanabe, T., Kimura, M., and Asakawa, S. (2006). Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). *Soil Biol. Biochem.* 38, 1264-1274.

Watanabe, T., Kimura, M., and Asakawa, S. (2007). Dynamics of methanogenic archaeal communities based on rRNA analysis and their relation to methanogenic activity in Japanese paddy field soils. *Soil Biol. Biochem.* 39, 2877-2887.

Watanabe, T., Kimura, M. and Asakawa, S (2009). Distinct members of a stable methanogenic archaeal community transcribe *mcrA* genes under flooded and drained conditions in Japanese paddy field soil. *Soil Biol. Biochem*. 41, 276-285.

Watanabe, A., Machida, N., Takahashi, K., Kitamura, S., and Kimura, M. (2004). Flow of photosynthesized carbon from rice plants into the paddy soil ecosystem at different stages of rice growth. *Plant Soil* 258, 151-160.

Watanabe, T., Wang, G., Lee, C. G., Murase, J., Asakawa, S., and Kimura, M. (2011). Assimilation of glucose-derived carbon into methanogenic archaea in soil under unflooded condition. *Appl. Soil Ecol*. 48, 201-209.

Weber, S., Stubner, S., and Conrad, R. (2001). Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. *Appl. Environ. Microbiol* 67, 1318-1327.

Weller, S., Kraus, D., Ayag, K. R. P., Wassmann, R., Butterbach-Bahl, K., and Kiese, R. (2014). Methane and nitrous oxide emissions from rice and maize production in diversified rice cropping systems. *Nutr. Cycl. Agroecosys.* (submitted).

Wu, X. L., Friedrich, M. W., and Conrad, R. (2006). Diversity and ubiquity of thermophilic methanogenic archaea in temperate anoxic soils. *Environ. Microbiol*. 8, 394-404.

Zinder, S. H. (1993). "Physiological ecology of methanogens," in *Methanogenesis* ed. J. G. Ferry (New York, NY: Springer US), 128-206.

Zinger, L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M., Huse, S. M., Welch, D. B.,*et al*. (2011). Global patterns of bacterial beta-diversity in seafloor and sea-water ecosystems. *PLoS ONE* 6:e24570. doi:10.1371/ journal.pone.0024570.

Chapter 4

Crop rotation of flooded rice with upland maize impacts the resident and active methanogenic microbial community

Björn Breidenbach[1], Martin B. Blaser[1], Melanie Klose[1] and Ralf Conrad [1¶]

[1]Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

Contributions:

B.B. designed the study and the sampling scheme, performed sampling, nucleic-acid extractions, T-RFLP analysis, qPCR analysis, analytical analysis (HPLC, pH, water content) and PCRs for 454 pyrosequencing (for all samples from 2011 wet; 2012 dry; 2012 wet; 2013 dry), evaluated the data, performed statistical analysis and wrote the manuscript.

M.B.B. designed the study and performed sampling.

M.K. performed nucleic-acid extractions, T-RFLP analysis, qPCR analysis and PCRs for 454 pyrosequencing (samples: 2013 wet).

R.C. designed the study and the sampling scheme, wrote the manuscript.

4.1 Abstract

Crop rotation of flooded rice with upland crops is a common management scheme allowing the reduction of water consumption along with the reduction of methane emission from paddy rice fields. The introduction of an upland crop into the paddy rice ecosystem leads to dramatic changes in field conditions (oxygen availability, redox conditions). However, the impact of this practice on the archaeal and bacterial community has scarcely been studied. Here, we provide a comprehensive study focusing on the crop rotation between flooded rice in the wet season and upland maize (RM) in the dry season in comparison to flooded rice (RR) in both seasons. The composition of the resident and active microbial communities was assessed by 454 pyrosequencing targeting the archaeal and bacterial 16S rRNA gene and 16S rRNA. The archaeal community composition changed dramatically in the rotational fields indicated by a decrease of anaerobic methanogenic lineages and an increase of aerobic *Thaumarchaeota*. Members of *Methanomicrobiales, Methanosarcinaceae, Methanosaetaceae, Methanocellaceae* were equally depressed in the rotational fields indicating influence on both acetoclastic and hydrogenotrophic methanogens. Contrary, members of Soil Crenarchaeotic Group, mainly *Candidatus* Nitrososphaera, were higher in the rotational fields possibly indicating increasing importance of ammonia-oxidation during drainage. In contrast minor effects on the bacterial community were observed. *Acidobacteria* and *Anaeromyxobacter* spp. were enriched in the rotational fields while members of anaerobic *Chloroflexi* and sulfite reducing members of *Deltaproteobacteria* were found in higher abundance in the rice fields. Quantitative PCR (qPCR) targeting the archaeal and bacterial 16S rRNA genes and 16S rRNA revealed a decrease of the resident bacterial and archaeal community during maize cultivation followed by increased growth during rice cultivation. Combining qPCR and pyrosequencing data revealed increased ribosomal numbers per cell for methanogenic species during crop rotation. This stress response, however, did not allow the methanogenic community to recover in the rotational fields during re-flooding and rice cultivation. In summary, the analyses showed that crop rotation with upland maize led to dramatic changes in the archaeal community composition whereas the bacterial community was only little affected.

4.2 Introduction

Rice is one of the most consumed staple foods worldwide and nourishes about 3 billion people (Maclean *et al*., 2002). With the anticipated increase in world's population the need for cultivating rice will increase (Van Nguyen and Ferrero, 2006). However, growing rice implies intensive water consumption (3,000 – 5,000 l/kg rice) as rice consumes up to 2-3 times more water per hectare than other crops (Tuong *et al*., 2005). Therefore, it is predicted that rice farmers will face "economic water scarcity" with increasing costs for irrigation and "physical water scarcity" as water supplies for irrigation shrink (Bouman *et al*., 2005). In contrast, maize does not require as much as water as rice and furthermore, is considered to be a cash crop, which already dominates the upland agricultural system in the Philippines (Kenmore and Flinn 1987; Bertomeu, 2012). Increasing demands of maize for fodder (poultry) and biofuel production (Weller *et al*., 2015a) along with a reduced water consumption may lead to increasing conversion from traditional rice–rice (wet–dry season) to rice–maize cropping management systems in tropical and subtropical Asia. Consequently, rice–maize systems are notably implemented today (Timsina *et al*., 2010).

In rice-maize crop rotations long-term submerged and anoxic soil conditions are changed to long-term aerated soil conditions accompanied by completely different redox conditions. Flooding of rice field soil causes a sequential depletion of the oxidants O_2, nitrate, ferric iron and sulfate that usually lasts several days (Ponnamperuma 1972; Conrad and Frenzel 2002). Throughout this period of changing redox processes CO_2 is the main product of degradation of organic matter. Afterwards, methanogenesis is the exclusive process in which organic matter is anaerobically degraded to CH_4 and CO_2. In upland soils, by contrast, CO_2 is the exclusive product of organic matter degradation. Moreover, upland soils quite often act as a sink for atmospheric CH_4 (Dutaur and Verchot 2007; Soussana *et al*. 2007). However, drained rice field soil apparently is not a sink for atmospheric CH_4 (Jäckel *et al*., 2001). In upland soils and drained soils CH_4 production occurs rather rarely, and if CH_4 is produced, production occurs in small anoxic microniches (Megonigal and Guenther 2008).

Particularly since the production of CH_4 in rice fields occurs under anaerobic conditions and the catalyzing microbial community is oxygen sensitive, crop rotations can have dramatic effects on the processes and organisms involved in methane production. However, archaeal communities were usually found to be rather stable in composition during stress events under field conditions. For example, after short term drainage or extended periods of managing rice fields as upland fields the archaeal community was only little affected (Krüger *et al*., 2005; Watanabe *et al*., 2006; Fernandez Scavino *et al*., 2013; **Chapter 3**, Breidenbach and Conrad, 2015). However, the expression of the activity of the methanogenic archaeal community changed over the season and with the flooding regime (Watanabe *et al*., 2007). By contrast, the bacterial community in rice field soil changed with time after flooding (Noll *et al*., 2005; Rui *et al*., 2009) and differed between oxic and anoxic zones (Shrestha *et al*., 2007). Furthermore, water-saving practices were shown to impact the bacterial community in rice field soil under field conditions (Ahn *et al*., 2014; Itoh *et al*., 2013).

Several management strategies have been developed to reduce water requirement for wetland rice fields such as alternate wetting and drying (Wassmann *et al*., 2000a, b), mid-season drainage (Wassmann *et al*., 2000b), or intermittent drainage (Yagi *et al*., 1996). All these strategies are based on restricted irrigation patterns during the cultivation of flooded rice. Thereby, short periods of drainage (weeks) allow the regeneration of inorganic electron acceptors (Ratering and Conrad, 1998). After re-flooding the sequential depletion of the oxidants is occurring again thereby suppressing methanogenic conditions until all the inorganic oxidants are depleted. Short term drainage strategies thus result in a repetition of the complete redox sequence as after the first flooding of the fields in the season.

In contrast, during crop rotations with upland crops innate irrigated rice fields are exposed to long periods (several months) of drainage during which the conditions are comparable to those in upland fields. This includes long-term aeration of the soil, which possibly causes oxygen stress for the inhabiting anaerobic microorganisms, and instead enhances the activity of aerobic microorganisms. Furthermore, the introduction of an allochthonous plant may impact the microbial community since rhizodeposition differs between plants (Klein *et al*., 1988; Marschner *et al*., 2001). Several studies showed that the

bacterial communities are affected by root exudation (Marschner *et al*., 2004; Haichar *et al*., 2008). Rice plants were also shown to support archaeal and bacterial lineages with plant derived carbon (Lu and Conrad, 2005; Pump *et al.*, 2014; Hernández *et al*., 2015; Zhu *et al*., 2014).

The present study was part of the multidisciplinary research project ”Introduction of non-flooded crops in rice-dominated landscapes and its impact on carbon, nitrogen and water cycles (ICON)” conducted at experimental fields of the International Rice Research Institute (IRRI) in the Philippines. During this study we found that the immediate effect of introducing drainage and maize cultivation to indigenous flooded rice fields were only minor (**Chapter 3**, Breidenbach and Conrad, 2015). Thereby, the effect of drainage seemed to be more pronounced than the shift in cropping from rice to maize. Field scale gas measurements showed a decrease in CH_4 emission upon maize cultivation (Weller *et al*., 2015a). However, the immediate effect of drainage and change of crop may not completely describe the response of the resident microbial community to such dramatic changes in field condition. Several questions arise, such as whether long term changes occur in the microbial community and if so, whether these changes are reversible.

We therefore made a comprehensive long-term study monitoring both archaeal and bacterial resident and active communities in response to crop rotation under field conditions. We hypothesized that the microbial community in rice field soil will be altered by crop rotation of rice (flooded soil) and upland maize (drained soil). Therefore, one crop rotation system (RM) with cultivation of irrigated rice in the wet season (summer) and upland maize in the dry season (winter) was compared to a control system with flooded rice cultivated in both seasons (RR). We investigated the microbial communities in the soil under field conditions over two years of crop rotation including five seasons. The microbial composition and abundance was assessed by fingerprinting with terminal-restriction length polymorphism (T-RFLP) and quantitative PCR (qPCR) targeting the archaeal and bacterial ribosomal 16S rRNA and 16S rRNA gene, respectively. In order to identify changes in the lower taxonomic groups, archaeal and bacterial 16S rRNA was targeted by 454 pyrosequencing.

4.3 Material and methods

4.3.1 Sampling site and sample processing

The sampling site was located at the International Rice Research Institute (IRRI) in Los Banos, Philippines. Detailed site description can be found in Heinz *et al*. (2013). This work was part of the interdisciplinary project "Introducing Non-Flooded Crops in Rice-Dominated Landscapes: Impact on Carbon, Nitrogen, and Water Cycles (ICON)". A detailed description of the field experiment can be found in Weller *et al*. (2015a). The experiment consisted of a flooded rice–maize crop rotation (maize-mix; RM) and a control with only flooded rice (rice-wet; RR). Briefly, we studied fields cultivated with either irrigated rice or upland maize at the reproductive growth phase of the plants over three years in both the dry season and the wet season. Fields under crop rotation (RM) were drained and managed as upland fields cultivating upland maize (variety: Pioneer P3482YR) in the dry season and flooded again and cultivated with rice (variety: NSIC Rc222) in the wet season. The control fields (RR) fields were flooded and cropped with rice both in the dry and the wet season with drainage in between. A detailed overview of the crop rotation system and the sampling time points is given in Table 4.1. Fields were operated in triplicates (RR: fields 3,6,9; RM: fields 4,7,10) and managed with conventional N-fertilization (rice: seeding 30 kg N/ha, 30 kg P_2O_5/ha, 30 kg K_2O/ha; at 28 and 55 days after seeding (DAS) 50 kg N/ha; maize: 30 kg N/ha, 50 kg P_2O_5/ha, 3 0 kg K_2O/ha; at 27-29 and 47-50 DAS 50 kg N/ha). In each of these fields we randomly selected three sampling plots of one square meter and sampled one soil core (5 cm diameter, 20 cm length) from each plot. The samples of the wet season 2011 were only generated from one field and sampled in triplicates (n=3). Soil cores were always taken in the vicinity of a plant (ca. 10 cm). The soil contained numerous fine roots and thus was most probably influenced by the plant roots. However, no attempts were made to separate a specific rhizospheric soil compartment. Subsequently, soil samples of 5 g were taken from the middle of the core (~ 10 cm depth), added to 10 mL RNAlater© solution (Life Technologies, Darmstadt, Germany), kept on ice and later stored at -20°C to ensure RNA stability. For further analysis (determination of soil variables), additional samples of 50 g were taken from the same soil core, homogenized and stored at -20°C.

4.3.2 Determination of soil variables

For the determination of soil water content small amounts of soil (1-5 g) were dried at 65°C for 3 days. The pH of the soil was analyzed following the DIN ISO 10390 protocol as described in **Chapter 3**, Breidenbach and Conrad (2015). Results are shown in Supplement Table 4.1. The soil texture was silt loam (**Chapter 3**).

4.3.3 Nucleic acid extraction

Nucleic acids were extracted from all replicates (n=9) following a modified version of the protocol of Bürgmann *et al* (2001) described in detail in **Chapter 3**, Breidenbach and Conrad (2015). Briefly, cells were mechanically disrupted by bead-beating in the presence of a phosphate buffer containing SDS. Total nucleic acids were purified using phenol/chloroform. Thereafter a subsample was treated with DNase in order to recover pure RNA after purification. Complete DNA removal was verified by failure to obtain a PCR amplification product of bacterial 16S rRNA genes with the purified RNA template using the conditions described below. cDNA synthesis was conducted using random hexamers in reverse transcription.

4.3.4 Quantitative polymerase chain reaction

The quantification of archaeal and bacterial 16S rDNA/rRNA was conducted using quantitative polymerase chain reaction (qPCR) based on a SYBRGreen approach. Therefor primer combinations Ba519f / Ba907r (Stubner, 2002) for bacterial and Ar364f (Burggraf *et al*., 1997) / Ar934br (Großkopf *et al*., 1998) for archaeal genes were used. A detailed protocol is given in **Chapter 3**.

4.3.5 454 Pyrosequencing

Tagged pyrosequencing of the bacterial and archaeal community was conducted using primer combinations F515/R806 (Bates *et al*., 2011) and Arch344F (Casamayor *et al*., 2002) / A934br (Großkopf *et al*., 1998), respectively. The forward primers were tagged with a unique 8-base pair barcode. Sequencing of the PCR products was done at the Max Planck Genome Centre in Cologne using a Roche 454 Genome Sequencer GS FLX+. One of the

triplicate samples from each field was randomly chosen and analyzed as representative for the field. Data analysis was performed using mothur software package version 1.31.2 (http://www.mothur.org/) following the standard operational procedure (SOP, Schloss *et al*., 2009). Sequence quality management and operational taxonomic units (OTU) analysis was conducted using UPARSE pipeline as described by Edgar (2013). Sequences derived from **Chapter 3**, RR and RM samples in dry season 2012, were integrated into the analysis (Supplement Table 4.2 to 4.5). Only microbial high-quality sequences with a minimum read length of 200 bp were used. Sequences that did not match the primer sequences and were smaller than 200 bp or contained any ambiguities were excluded from further analysis. After denoising, sequences were aligned against the SILVA bacteria 16S rRNA gene database using the naïve Bayesian classifier (Schloss *et al*., 2011; Wang *et al*., 2007; Pruesse *et al*., 2007). Sequences which were not assigned to bacteria or respectively archaea were discarded. OTUs were defined using a distance matrix with 3% dissimilarity (Zinger *et al*., 2011). Further analyses including rarefaction curves, species richness and diversity indices were conducted as described in the SOP pyrosequencing pipeline (Schloss *et al*., 2011). An overview of the number of sequences retrieved and the accession numbers of the submitted sequences can be found in Supplement Tables 4.2 to 4.5.

4.3.6 Statistical analysis

Statistical analyses were conducted in R version 2.14.1 (R Development Core Team, 2011). Analysis of variance (ANOVA), Hellinger transformation and principal component analysis (PCA) were done with package vegan version 2.0.5 (Oksanen *et al*., 2012). In order to identify OTUs which were detected in all samples (core) and OTUs which were specific for one sample (unique) venn diagrams were used. These venn diagrams were created using web based tool developed by Bioinformatics & Evolutionary Genomics department of the University Gent (http://bioinformatics.psb.ugent.be/webtools/Venn/). Heatmaps representing the relative sequence abundance of bacterial OTUs between samples were constructed with package gplots (Warnes, 2011). PCA was performed using Hellinger transformed OTU abundance and resulted in PC1, PC2 and PC3 explaining 13%, 10% and 7% of the variance for bacterial 16S rDNA and PC1, PC2 and PC3 explaining 17%, 10% and 6% for bacterial 16S rRNA, respectively. The OTUs explaining most of the differences between samples

were defined as the OTUs with the highest loadings. For 16S rDNA 13 OTUs of PC1, 10 OTUs of PC2 and 7 OTUs of PC3 were chosen to construct the heatmap (Abdi and Williams, 2010; Deng *et al*., 2014). For 16S rRNA 17 OTUs of PC1, 10 OTUs of PC2 and 6 OTUs of PC3 were chosen. Finally, a heatmap was constructed using the selected OTUs.

4.4 Results

4.4.1 Archaeal and bacterial 16S rDNA/rRNA copy numbers

In order to quantify archaea and bacteria in the flooded rice (RR) and the rice-upland maize crop rotation (RM) we used quantitative PCR (qPCR) targeting the archaeal and bacterial 16S ribosomal RNA (16S rRNA) and their genes (16S rDNA). Copy numbers of archaeal and bacterial 16S rDNA and rRNA were quantified at three wet and two dry seasons in the differently cropped fields (Figure 4.1). Both resident (16S rDNA) archaeal and bacterial abundances did not show large differences between the seasons (dry, wet) in the RR fields (Figure 4.1A, C; RR). However, variations between the seasons were observed for the active community (16S rRNA) (Figure 4.1B, D), also seen by the different ratios of 16S rRNA/rDNA copies (Supplement Figure 4.1). In comparison to RR fields, archaeal and bacterial 16S rDNA copy numbers in RM fields were always lower during the dry season when cropped with maize (Figure. 4.1).

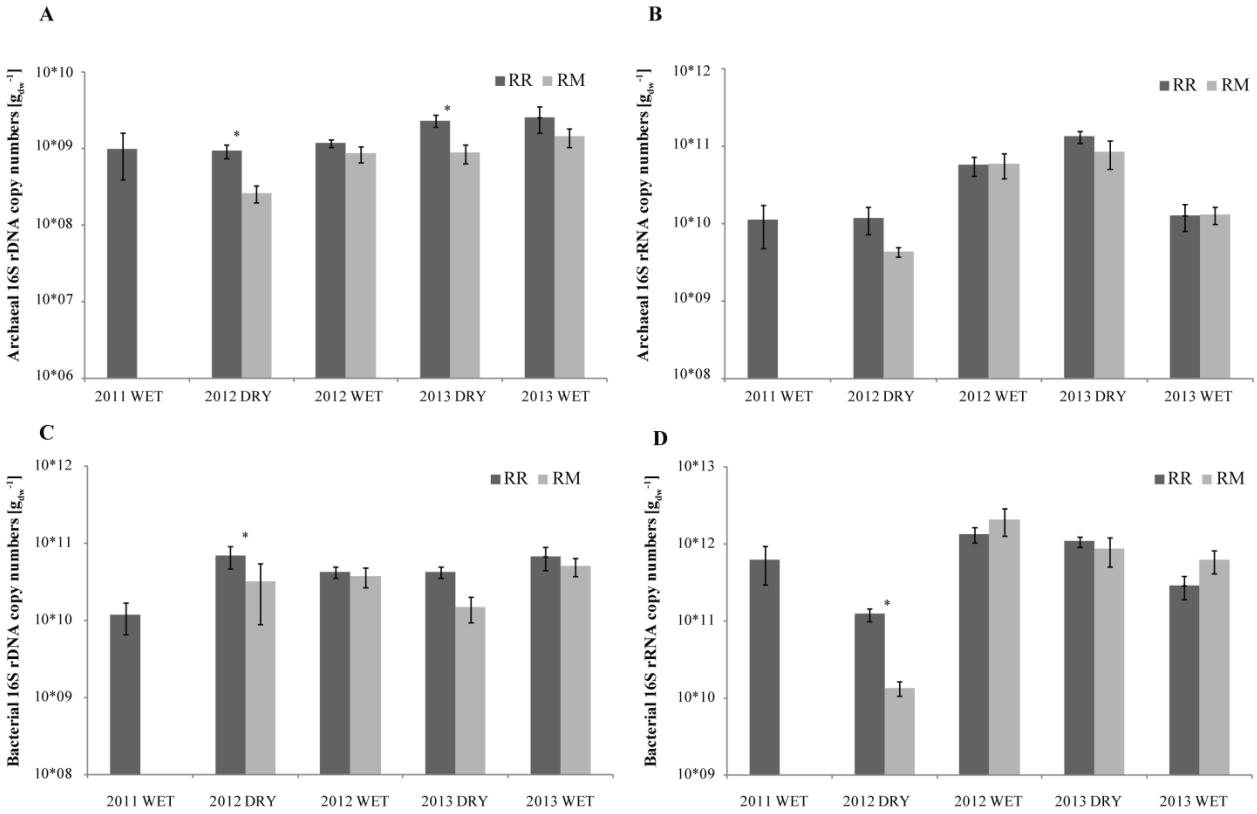


Figure 4.1. Ribosomal 16S rDNA and rRNA copy numbers quantified using qPCR. Abundance of archaeal 16S rDNA and rRNA (**A**, **B**) and bacterial 16S rDNA and rRNA (**C**, **D**) in control rice fields (RR, dark grey) and rice-upland maize crop rotation (RM, light grey) during dry (dry) and wet (wet) season. Asterisks indicate significant difference between RR and RM (ANOVA, $P < 0.05$) (mean ± SE, n=3 (2011 Wet), n=9 (all others)).

Table 4.1 Overview of cropping system and sampling time points for the investigated field types.

	2011	2012		2013	
Field type	*Wet (July)*	*Dry (April)*	*Wet (August)*	*Dry (February)*	*Wet (July)*
RR	Rice[¶]	Rice[¶]	Rice[¶]	Rice[¶]	Rice[¶]
RM	Rice[*]	Maize[¶]	Rice[¶]	Maize[¶]	Rice[¶]

* = not sampled, ¶ n= 3

The decrease of the resident archaeal and bacterial community was significant during the first maize cultivation in the dry season 2012 (ANOVA, $P < 0.05$, Figure 4.1A, C). The number of archaeal 16S rDNA was again significantly lower under maize cultivation in the following dry season 2013 (ANOVA, $P < 0.05$, Figure 4.1A), but the decrease in bacterial abundance in dry season 2013 was not statistically significant (Figure 4.1C). In contrast to the resident archaea and bacteria (16S rDNA), the numbers of the active members (16S rRNA) were similar for RR and RM fields, except the bacterial 16S rRNA copies during the first dry season 2012 in the maize fields, which were significantly lower in RM than in RR (Figure 4.1C, D).

4.4.2 Pyrosequencing of archaeal 16S rDNA and rRNA

Pyrosequencing targeting archaeal16S rDNA and rRNA was conducted in order to identify the resident respectively the active archaeal phylotypes in the Philippine rice field soil and to monitor the influence of season and crop rotation on the archaeal community composition. The communities were dominated by sequences of members of the classes Soil Crenarchaeotic Group and *Methanomicrobia* (Supplement Figures 4.3, 4.4). The class Soil Crenarchaeotic Group consisted mainly of Unclassified Soil *Crenarchaeota* and of members of the *Candidatus* Nitrosophaera, indicating that these *Crenarchaeota* can probably be classified as *Thaumarchaeota* (Pester *et al*., 2011). The class *Methanomicrobia* mainly consisted of *Methanosarcinales* with minor (<5%) contributions by *Methanomicrobiales* and *Methanocellales* (Supplement Figure 4.3, 4.4). *Methanobacteriales* (class *Methanobacteria*) were also present in low amounts (<5%).

The relative proportion of the resident community of *Euryarchaeota* (mainly *Methanosarcinales*) versus *Crenarchaeota* (presumably mainly *Thaumarchaeota*) stayed relatively constant with time in the RR fields, but changed in the RM fields (Figure 4.2A, B). Thus, the relative abundance of the resident *Crenarchaeota* strongly increased in the wet season 2012 following the first maize cultivation. At the same time, the relative abundance of the resident *Euryarchaeota* decreased. The relative increase of *Crenarchaeota* and decrease of *Euryarchaeota* further intensified in the dry season 2013. In contrast to the resident communities, the active communities (16S rRNA) of *Crenarchaeota* and *Euryarchaeota* did not exhibit such a distinct behavior (Figure 4.2C, D), although the relative abundance of *Crenarchaota* was always higher than that of *Euryarchaeota* in RM soil during the dry season, i.e. when the fields were cultivated with upland maize.

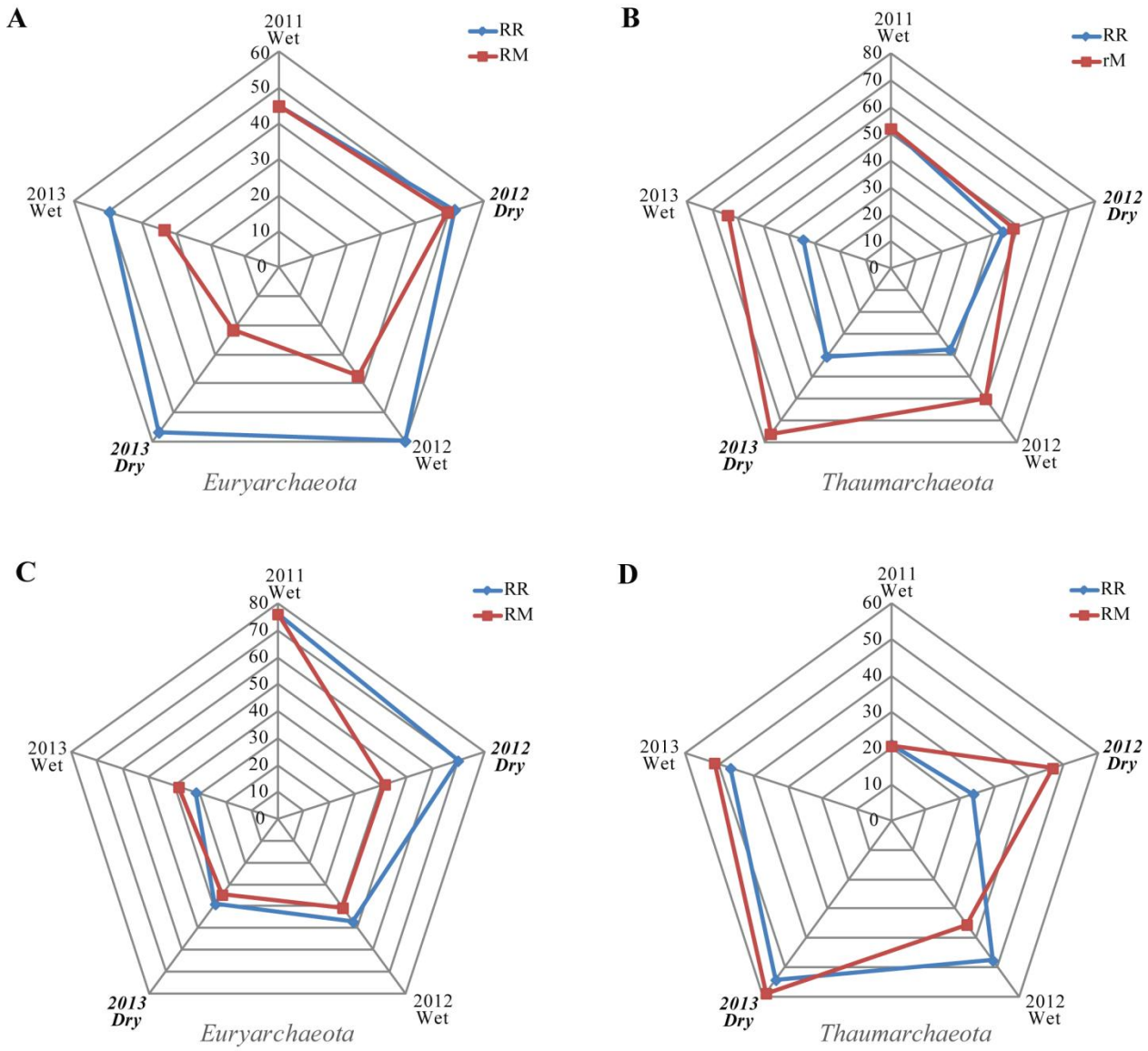


Figure 4.2 Radar plot of archaeal phyla represented as relative abundance of sequences from 454 pyrosequencing. Temporal dynamics of resident (**A**, **B**) and active (**C**, **D**) *Euryarchaeota* (**A**, **C**) and *Crenarchaeota* (**B**, **D**) in control rice fields (RR; blue) and rice-upland maize crop rotation (RM; red) during dry (dry) and wet (wet) season are shown.

In order to analyze the dynamics within the resident (rDNA) and active (rRNA) archaeal community, the 50 most abundant OTUs, which represented 64 - 83% of the sequences, were identified (Supplement Table 4.6). The detailed taxonomic classification of the top 50 archaeal OTUs is given in Supplement Table 4.7. The compositional change of the archaeal community over the season and between RR and RM fields is shown in Figure 4.3. Therefore the taxonomy of the archaeal OTUs is represented in on lowest taxonomic level.

The resident archaeal community was dominated by the Soil Crenarchaeotal Group, of which the relative abundance increased in the RM versus the RR fields from wet season 2012 until wet season 2013 (ANOVA, $P < 0.05$, Figure 4.3). While the Soil Crenarchaeotal Group showed constant relative abundance (~10%) in the RR fields, an increase in abundance in the RM fields with up to ca. 50% during dry season 2013 was observed (Figure 4.3A). By contrast, GOM Arc I (*Methanosarcinales*) decreased in the RM fields during dry season 2013 in comparison to the RR fields (ANOVA, $P < 0.05$, Figure 4.3A). Some methanogenic phyla showed statistically higher abundances in the RR fields than in the RM fields, for example *Candidatus* Methanoregula (wet, 2011) and *Methanosaeta* (dry, wet 2013) (ANOVA, $P < 0.05$, Figure 4.3A). In addition, several trends, albeit not statistically significant, are worth to be mentioned. While thaumarchaeotal *Candidatus* Nitrososphaera was higher in the RM fields, the methanogenic lineages *Methanobacterium* and *Methanosarcina* increased in abundance during the dry seasons (2012, 2013) in the RR fields.

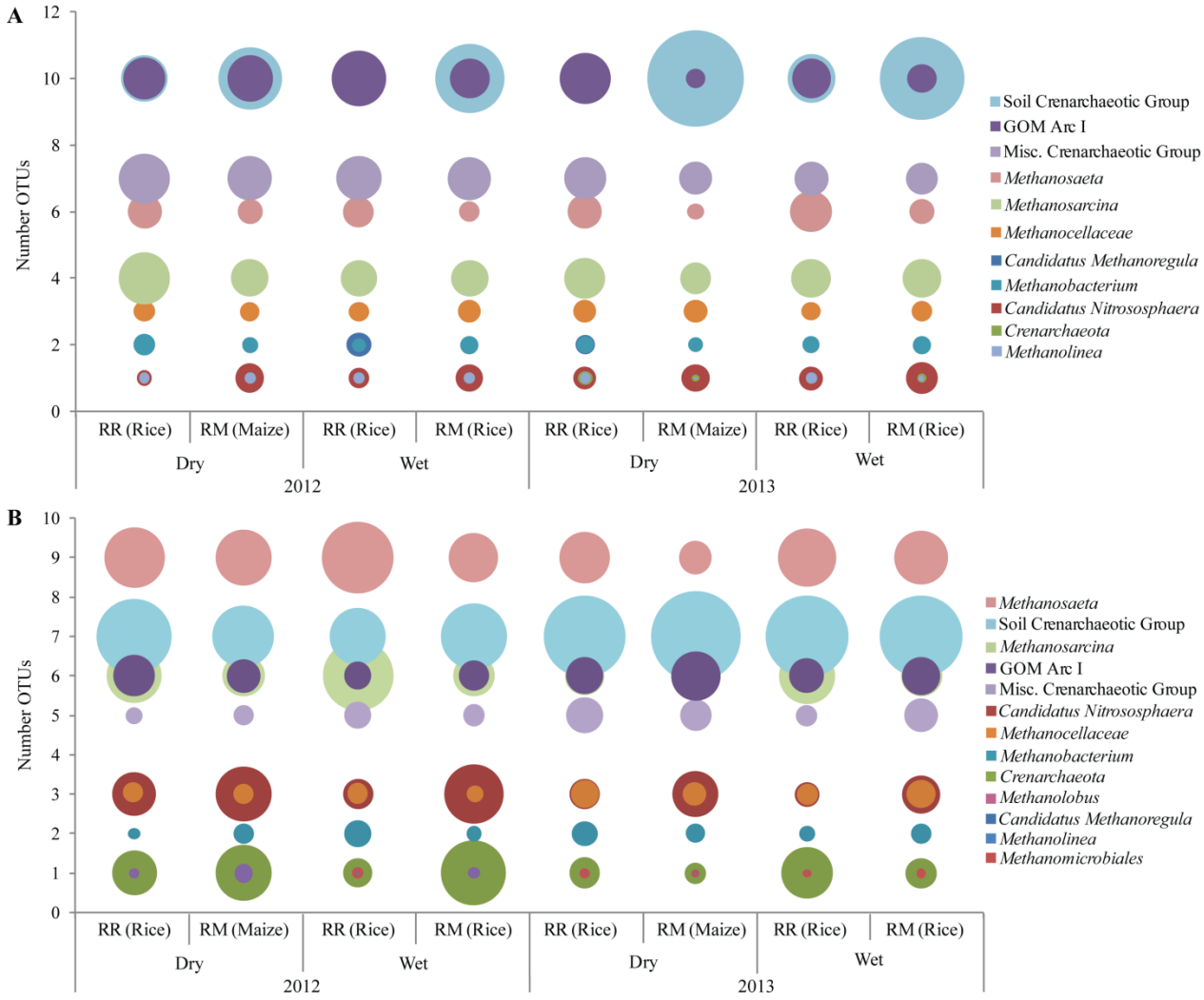


Figure 4.3 OTU based relative abundance of the archaea. The top 50 OTUs, based on relative abundance, were grouped according to their phylogenetic assignment for 16S rDNA (**A**) and 16S rRNA (**B**). Bubble plot of the archaeal OTUs in control rice fields (RR) and rice-upland maize crop rotation (RM). Bubble represents phylogenetic group and bubble size the relative sequence abundance (0.3 - 60%). Phylogenetic groups were ordered according to the number of OTUs they represent**.**

The active archaeal community was dominated by the same species as the resident community, namely Soil Crenarchaeotic Group and *Methanosaeta* (Figure 4.3B). Here, several trends, albeit not statistically significant, are worth to be mentioned: *Crenarchaeota*, *Candidatus* Nitrososphaera and Misc. Crenarchaeotic Group (probably belonging to *Thaumarchaeota*) showed increased relative abundances in the RM fields (Figure 4.3B). However, methanogenic groups like *Methansaeta* and *Methanosarcina* were seemingly more active in the RR than RM fields (Figure 4.3B).

The ratio between 16S rRNA/rDNA of the different phylogenetic groups was determined by multiplying the relative sequence abundances with the corresponding qPCR data. The 16S rRNA/rDNA ratios all increased from dry season 2012 until dry season 2013 and then decreased again, however only some statistically significant (ANOVA $P < 0.05$; Figure 4.4). Interestingly, the 16 rRNA/rDNA ratios of the classes *Methanobacteria* and *Methanomicrobia* were always higher in the RM than RR fields from wet season 2012 onwards (Figure 4.4A), while it was opposite for the Soil Crenarcheotic Group, which were higher in the RR than RM fields during wet season 2012 and dry season 2013 (Figure 4.4B). Deeper phylogenetic analysis revealed that among the *Methanobacteria* and *Methanomicrobia* it was the groups of *Methanobacteriaceae*, *Methanocellaceae*, *Candidatus* Methanoregula and genus *Methanolinea* that exhibited the relative increase of 16S rRNA/rDNA ratios in the RM fields (Supplement Figure 4.5B, C). Among the Soil Crenarchaeotic Group there was not a particular phylogenetic group that showed such increase in the RR fields (Supplement Figure 4.5A).

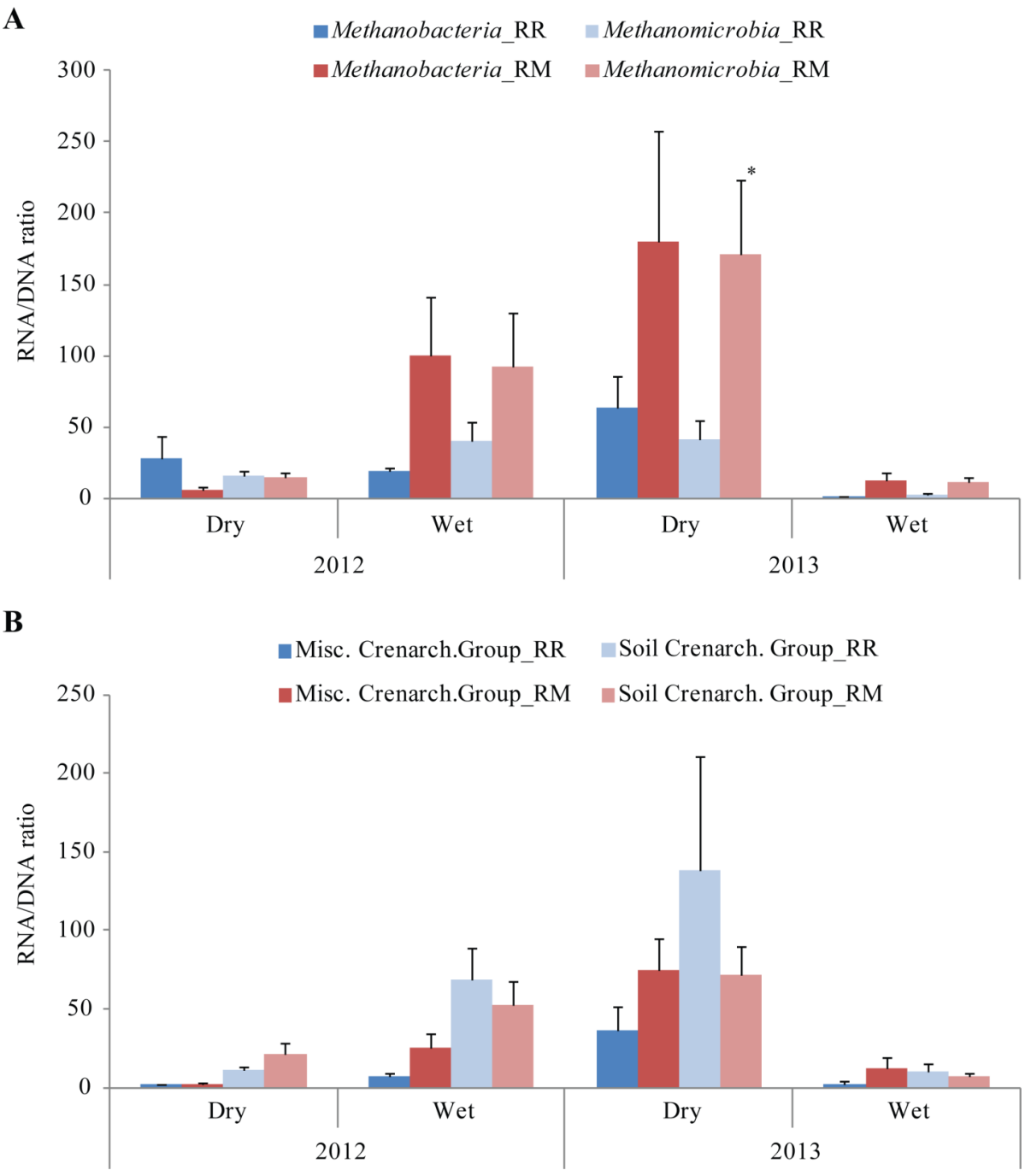


Figure 4.4 Ratio of ribosomal 16S rRNA and rDNA copy numbers multiplied by the relative sequence abundance of archaeal classes. Euryarchaeotic (**A**) and thaumarchaeotic (**B**) classes derived ratios are shown for dry and wet season 2012 and 2013 in the fields with control rice cultivation (RR; blue) and the flooded rice-upland maize crop rotational fields (RM, red). Columns represent mean and bars standard errors of n=3. Asterisk represents statistically significant differences at one particular season (ANOVA, $P < 0.05$).

4.4.3 Pyrosequencing of bacterial 16S rDNA and rRNA

The pyrosequencing for monitoring bacterial phylotypes in the rice-upland maize crop rotation (RM) and the wetland rice control (RR) targeted the bacterial 16S rDNA (resident bacteria) and rRNA (active bacteria). Both the resident and active bacterial communities were dominated by *Proteobacteria* and *Acidobacteria*. The resident community was in addition dominated by *Chloroflexi* (Supplement Figure 4.5). The pyrosequences were grouped into OTUs, of which only 2-7% were unique, while 33-75% were found in all replicate samples, i.e. were core OTUs for a particular type of field and season (Supplement Table 4.6). The core OTUs of the resident (Figure 4.5A) and the active (Figure 4.5B) communities also show the dominance of *Deltaproteobacteria*, *Acidobacteria* and *Chloroflexi* (only resident community). Among these major taxa there was not much change in their relative abundance from dry season 2012 to wet season 2013 and there were no statistically significant differences (ANOVA, $P > 0.05$) in RM versus RR fields (Figure 4.5) both among resident and active bacterial communities. Analysis of individual phylotypes among the resident bacterial community showed seasonal differences within the *Acidobacteria*, *Chloroflexi* and *Deltaproteobacteria*, for example the *Anaerolineaceae* and *Anaeromyxobacter* in the RR fields (ANOVA, $P < 0.05$; Figure 4.6A), and the *Caldilineaceae* in the RM fields (ANOVA, $P < 0.05$; Figure 4.6A). A similar pattern was seen in the communities of active bacteria (Figure 4.6B).

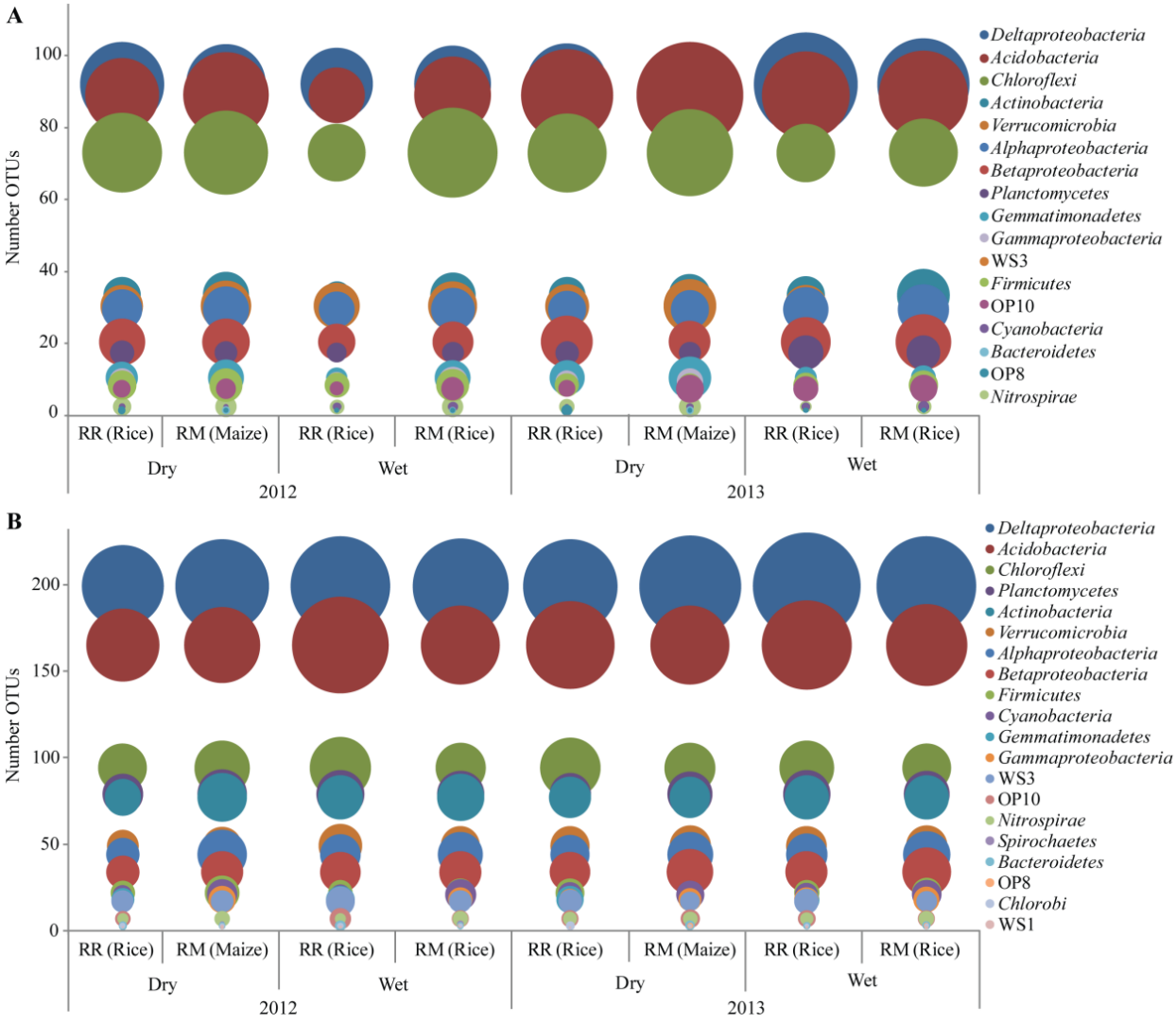


Figure 4.5 OTU based relative abundance of the bacteria. OTUs detected in all fields (core) were grouped according to their phylogenetic assignment for 16S rDNA (**A**) and 16S rRNA (**B**). Bubble plot of the archaeal OTUs in control rice fields (RR) and rice-upland maize crop rotation (RM). Bubble represents phylogenetic group and bubble size the relative sequence abundance (0.5 - 20%). Phylogenetic groups were ordered according to the number of OTUs they represent**.**

Since it was at the first glance not possible to identify specific bacterial phylotypes that were impacted by crop rotation, we used two approaches, i.e., (i) a PCA-based approach and (ii) a hypothesis driven approach. In the PCA-based approach the OTUs with the highest loadings on the major 3 PCA axes were chosen and represented in a heatmap (see Material and Methods, Figure 4.7). The selected OTUs representing the resident bacterial community showed clustering dependent on year and field type (Figure 4.7A). Most of these OTUs were found in similar relative abundance in both field types. However, in both years OTUs assigned to the *Acidobacteria* were more abundant in the RM fields while *Deltaproteobacteria* were higher in the RR fields. OTUs assigned as *Cystobacteraceae* showed increased abundance in wet season 2013 for both field types. The OTUs representing the active bacterial community showed clustering again among the same phylotypes, but with *Deltaproteobacteria* especially in RM fields in 2013 but without preference for dry or wet season (Figure 4.7B).

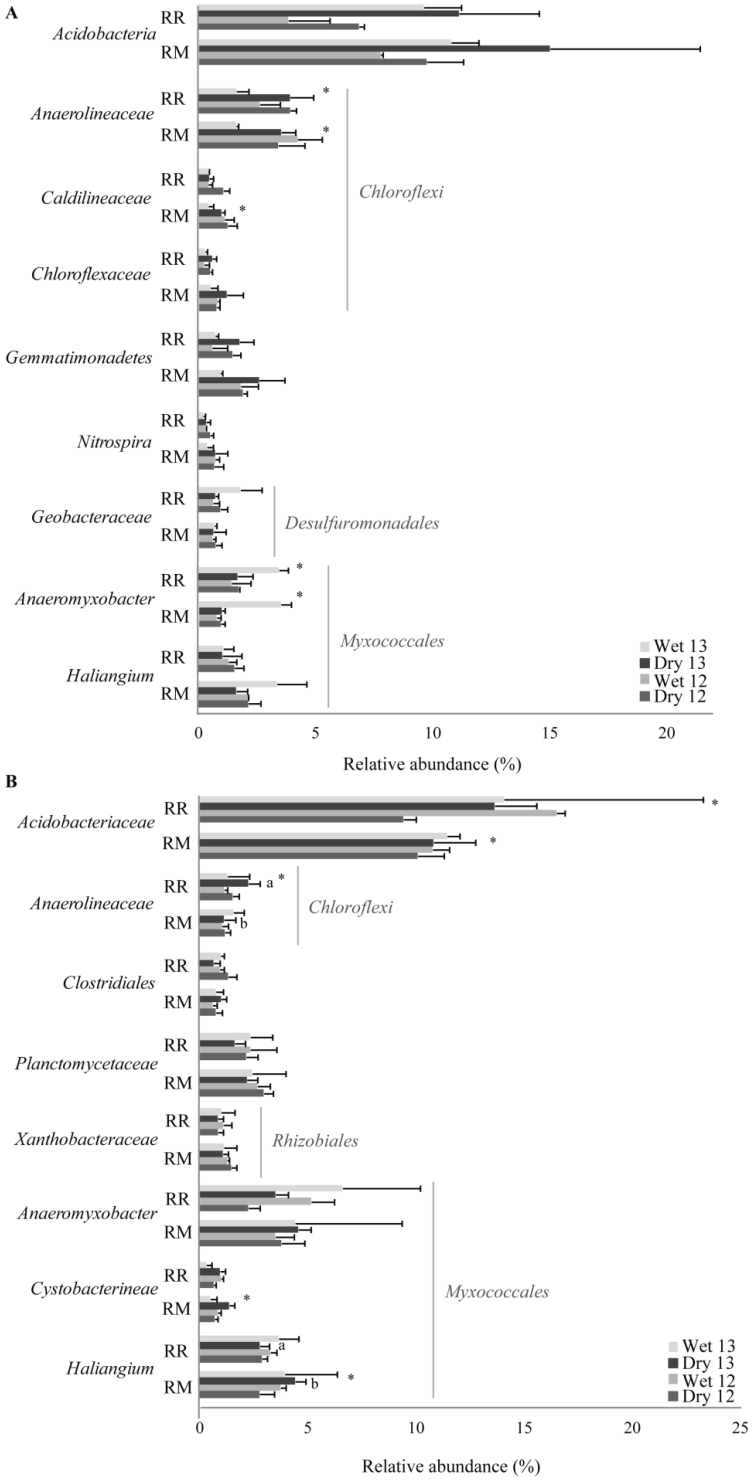


Figure 4.6 Individual phylogenetic groups out of the bacterial core OTUs. Phylogenetic groups are displayed based on 16S rDNA (**A**) and 16S rRNA (**B**) for the control rice fields (RR) and the rice-upland maize crop rotation (RM). Columns represent mean and bars standard deviations of n=3. Asterisk represents statistically significant differences within a field type over the seasons and letters between the field types at one particular season (ANOVA, $P < 0.05$).

In the hypothesis driven approach *Desulfobacterales* and *Syntrophobacterales* were analyzed, as they had been hypothesized to be potential syntrophic partners for methanogenic archaea (Itoh *et al*., 2013). In fact, these phyla exhibited a higher relative abundance in the RR than the RM fields (Figure 4.8A). However only some were statistically significant (ANOVA $P< 0.05$). Furthermore, *Bacilalles* and *Clostridiales* out of Firmicutes were analyzed, as they had been hypothesized to be favoured during upland soil conditions in crop rotations due to their ability to form endospores (Fernandez Scavino *et al*., 2013). Here, *Bacilalles* showed a higher relative abundance in the RM than the RR fields, however not statistically significant (ANOVA $P> 0.05$; Figure 4.8B). In addition, *Planctomycetes* were analyzed, as they several members able to perform anaerobic ammonium oxidation (annamox) had been hypothesized to be potential syntrophic partners for anaerobic methane oxidizers out if the archaea (Haroon *et al*., 2013) which in fact were found in higher abundance in the only rice fields. Here, *Planctomycetes* did not shown differences between the rotational and the rice fields, however not statistically significant (ANOVA $P> 0.05$; Figure 4.8C).

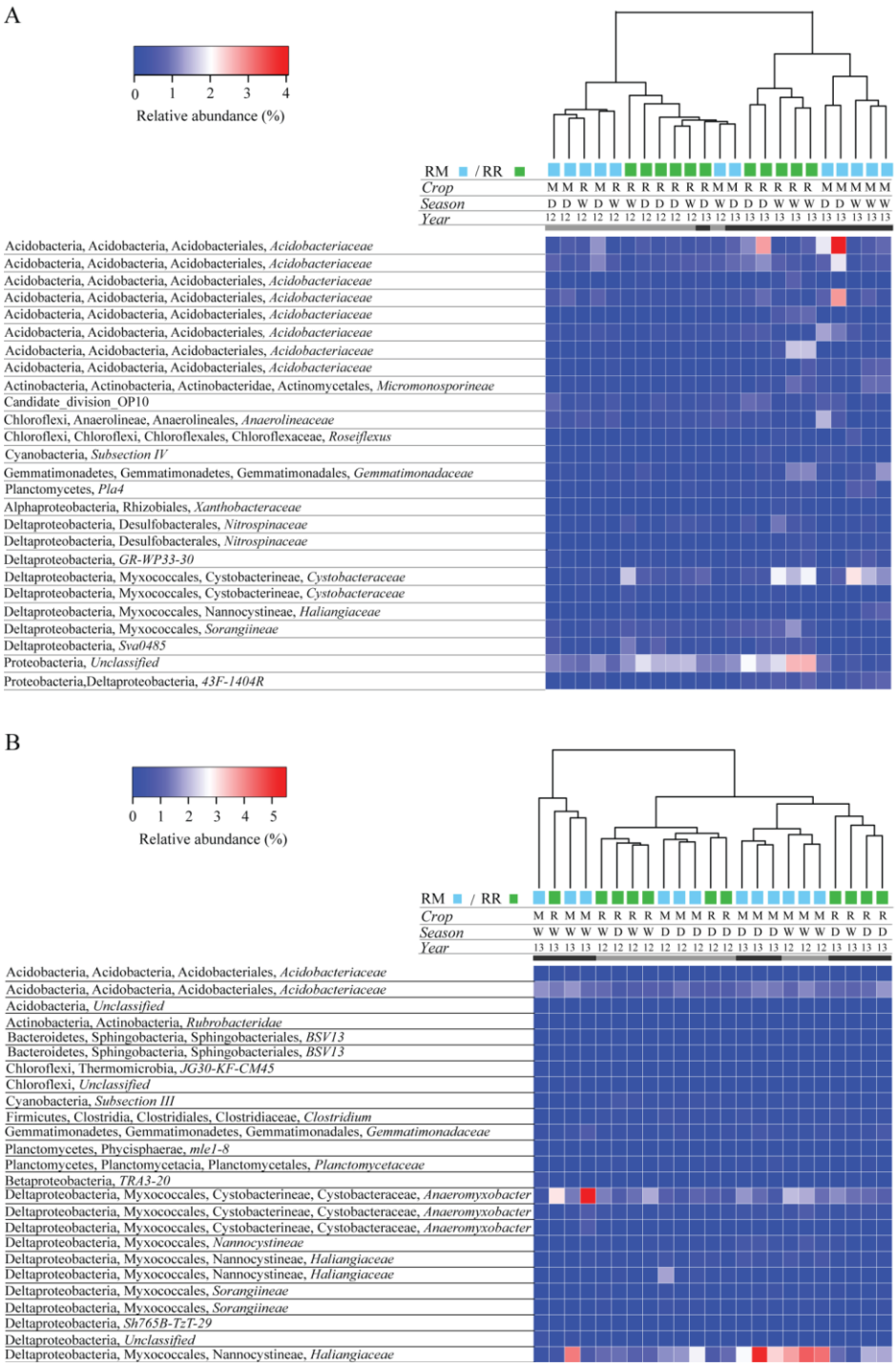


Figure 4.7 Heatmap showing the relative abundance of selected bacterial OTUs. OTUs based on 16S rDNA (**A**) and 16S rRNA (**B**) representing the resident and active bacterial community are displayed. The samples are clustered according to Bray-Curtis distances. The colors correspond to the relative sequence abundance of the OTUs, as indicated by the color legend. The taxonomy of each OTU is provided to the lowest-level achieved during the classification. Unclassified OTUs were excluded from the analysis. Samples from control rice fields (RR) and rice-upland maize crop rotation (RM) were represented and abbreviations specify crop maize (M) and rice (R), season dry (D) and wet (W), and year 2012 (12, grey) and 2013 (13, black).

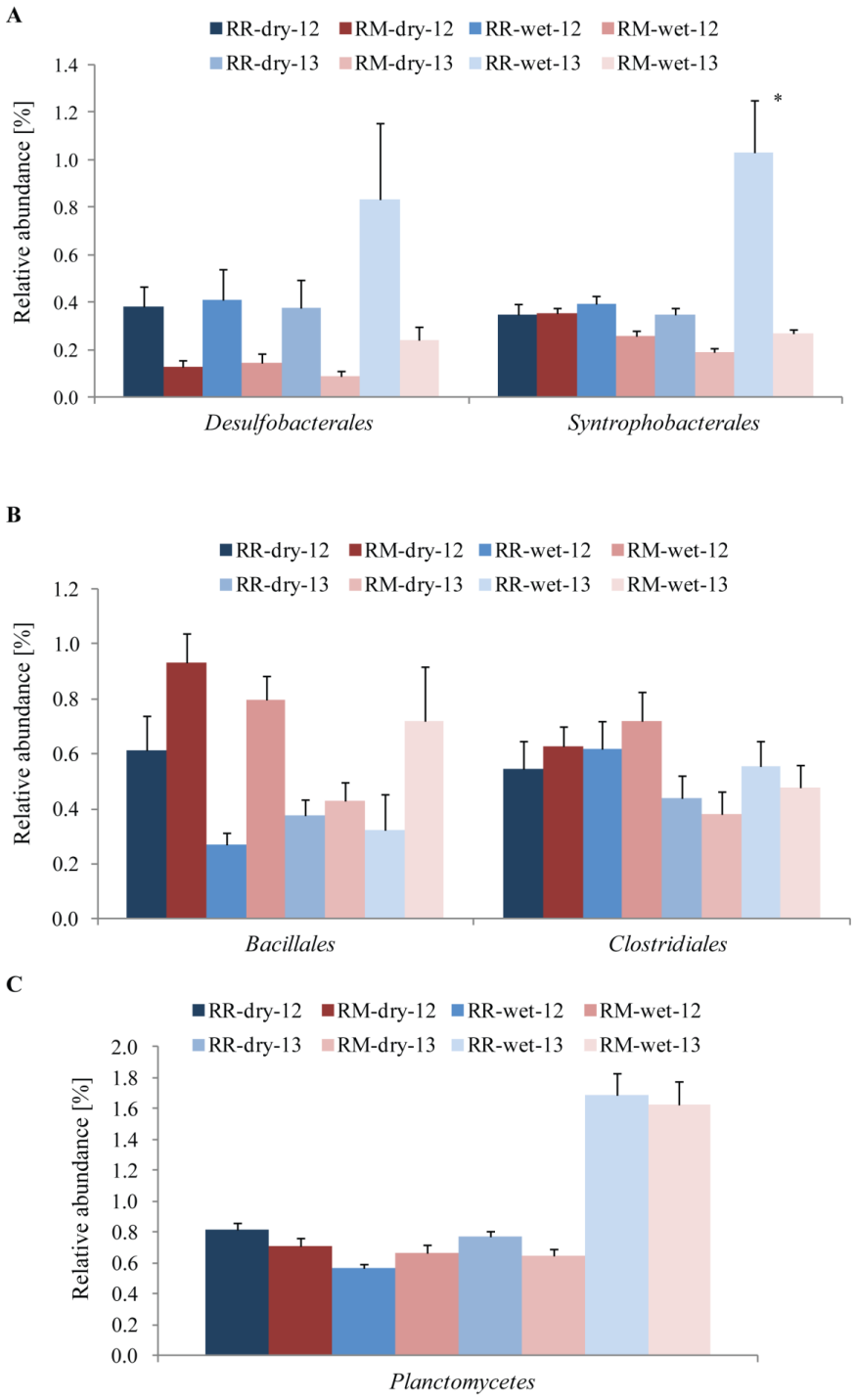


Figure 4.8 Relative abundance of selected bacterial (16S rDNA) core OTUs. OTUs assigned as *Desulfobacterales* and *Syntrophobacterales* (**A**), *Bacilalles* and *Clostridiales* (**B**) as well as *Planctomycetes* (**C**) are displayed. OTUs were obtained in control rice fields (RR) and rice-upland maize crop rotation (RM) during dry (dry) and wet (wet) season 2012 (12) and 2013 (13). Bars represent standard deviations of n=3. Asterisk represents statistically significant differences at one particular season (ANOVA, $P < 0.05$).

4.5 Discussion

Our study showed that introduction of crop rotation to irrigated rice cultivation resulted in a pronounced change in the composition of the communities of resident and active Archaea, but only in a minor change in those of the Bacteria. These changes were manifested not immediately after the first cultivation of upland maize, but established in the subsequent seasons, when irrigated rice cultivation in the wet season was alternated with upland maize cultivation in the dry season.

4.5.1 Impact of crop rotation on the archaeal community

Whereas the abundance of the resident Archaea was decreased during maize cultivation and recovered during re-flooding and rice cultivation, the archaeal community composition was affected more persistently. The community composition in the crop rotational fields diverged in comparison to that in the irrigated rice fields in the wet season (2012) after maize cultivation, when the fields were re-flooded and cultivated with rice. Notably, the abundance of non-methanogenic *Crenarchaeota*/*Thaumarchaeota* increased while the methanogenic *Euryarchaeota* decreased. This trend was not reversible during the following season and even strengthened at the second maize cultivation in dry season 2013. Along with the decreasing relative abundance of methanogens in the present study, significantly lower CH_4 emissions were observed in the crop rotational fields than in the control rice fields even after re-flooding and cultivation of irrigated rice (Weller *et al*., 2015b, in preparation). The annual emissions were found to be reduced to about 25% of the ones from irrigated rice fields. In contrast to our observations it was previously suggested that the methanogenic community can recover after periods of upland crop cultivation (Eusufzai *et al*., 2010). However, our observations are consistent with reports of lower numbers of resident and active archaea in drained versus flooded rice fields in Japan (Itoh *et al*., 2013) and with decreasing numbers of methanogens in Chinese fields during a rice-soybean rotation which never recovered to the level of the control rice fields without rotation (Liu *et al*., 2015).

The relative increase of *Crenarchaeota/Thaumarchaeota* was due to increase of members of the Soil Crenarchaeotic Group represented by *Candidatus* Nitrososphaera. Itoh

et al. (2013) also showed increased relative abundance of *Candidatus* Nitrososphaera related clones in drained rice field soil in comparison to flooded soils. Only recently, the first isolate, *Nitrososphaera viennensis,* was described, also proposing the novel genus *Nitrosophaera* within the novel family *Nitrosospharaceae* and novel order *Nitrososphaerales* (Stieglmeier *et al*., 2014). *Nitrososphaera viennensis* is an aerobic ammonia-oxidizing archaeon, thus suggesting that ammonia-oxidizing archaea become increasingly important in rice-maize rotational fields. Their increased importance may explain the increased N_2O emissions observed during the dry season in the maize fields by members of the ICON group (Weller *et al*., 2015b, in prep.).

Contrary to the thaumarchaeotal species, methanogenic *Euryarchaeota* (*Methanosaeta, Candidatus* Methanoregula, *Methanobacterium* and *Methanosarcina*) were found in lower relative abundance in the crop rotational fields indicating unfavourable conditions for these strict anaerobes in the drained fields. Thus, both acetoclastic and hydrogenotrophic methanogens were affected by crop rotation in the Philippine rice field soil. Other studies reported negative effects of drainage on the acetotrophic methanogenic community (Krüger *et al.*, 2001; Zhang *et al.*, 2012), but also on the hydrogenotrophic community (Itoh *et al*., 2013). There are also controversial results concerning the total methanogenic community, as several studies showed significant effects (Ma and Lu, 2011; Watanabe *et al*., 2013; Itoh *et al*., 2013), whereas other studies found that the archaeal communities were only little affected by crop rotation with upland crops (Asakawa and Hayano, 1995; Watanabe *et al*., 2006, 2009; Fernandez Scavino *et al*., 2013; **Chapter 3**, Breidenbach and Conrad, 2015). These differences are not easily explained, since comparison of different field experiments with their intrinsically complex conditions is notoriously difficult. We would like to emphasize that the effects of introduction of crop rotation on the methanogenic communities were relatively little and delicate. It is noteworthy that the discussed rotations differed in substantial aspects from each other, to name some rotation history, soil type/texture and plant species. The latter was maize in the present study while the Japanese rice was rotated with wheat (Asakawa and Hayano, 1995; Watanabe *et al*., 2006, 2009) and in the Uruguayan rotations pasture was used (Fernandez Scavino *et al*., 2013). Since it is known that plants influence the microbes in the soil e.g. via rhizodeposition and different plants select for different microbial communities (e.g.:

Marschner *et al*., 2004; Haichar *et al*., 2008) it may possible that the maize plant as a more pronounced influence on the microbes than pasture or wheat. Additional soil type may explain differences. Watanabe *et al*. (2006) suggested that the methanogenic communities in differed between two field location as result of different soil types described by dissimilarities in temperature and contents of total carbon and nitrogen. Further the history of crop rotation, meaning time from the first crop change to upland conditions, may explain the observed differences. To our knowledge the study of Breidenbach and Conrad (2015) (**Chapter 3**) followed by the present study are the first studies investigating the effect of crop rotation directly from the time point of the first rotation. In contrast, Asakawa and Hayano (1995) sampled the first time during the second upland phase and wheat cultivation and the fields studied by Watanabe *et al*. (2006, 2009) undergone the rotation pattern since 1963. Liu *et al*. (2015) reported that the methanogens differed in abundance and the composition between the rotational fields and the control rice fields, but within the rotational fields the numbers and community composition were unaltered regardless of managing the fields as flooded rice field or as upland soybean fields. Together with the results obtained in the present study this may indicate that the microbial community has first to adept to the stress of crop rotation before the archaeal and especially the methanogenic community reveals the relative stability observed in rice-wheat and pasture-rice crop rotations.

In the present study sequences assigned as GOM Arc I were found in all treatments and decreased significantly with upland conditions. GOM Arc I was formerly known as ANME-2d because of its phylogenetic relation to the anaerobic methanotrophs ANME-2 (Mills *et al*., 2005; Martinez *et al*., 2006). Recently, Haroon *et al*. (2013) identified members of ANME-2d capable of anaerobic methane oxidation (AOM) and proposed the name *Candidatus* Methanoperedens nitroreducens for the ANME-2d lineage. These organisms oxidize CH_4 through reverse methanogenesis using nitrate as terminal electron acceptor (Haroon *et al*., 2013). The thereby produced nitrite is reduced to dinitrogen gas by anaerobic ammonium-oxidizing bacteria (*Planctomycetes*) in a syntrophic interaction. However, here the *Planctomycetes* did not shown differences between the field types. Recently, high abundances of AOM bacteria were reported in a Chinese rice field (Zhou *et al*., 2014). As GOM Arc I/ANME-2d were found in relatively high relative abundance in Philippine and

South Korean rice field soil (**Chapter 3**, Breidenbach and Conrad, 2015; Ahn *et al*., 2014) we speculate that AOM conducted by Archaea has been underestimated in rice fields.

Despite the changes in relative abundance during crop rotation the full range of different methanogenic taxa (members of: *Methanomicrobiales, Methanosarcinaceae, Methanosaetaceae, Methanocellaceae*) was still present in the soil irrespectively of season, crop and water management. Some methanogenic groups were apparently not affected at all, e.g., *Methanocellacea* and *Methanosarcinaceae*. These groups were also reported in other studies to be present in rice field soil despite drainage or rotation with upland crops (Liu *et al*., 2015; Watanabe *et al*., 2009; Itoh *et al*., 2013; Fernandez Scavino *et al*., 2013). Both, *Methanosarcina* spp. and *Methanocella* spp. are conspicuous residents of dry and aerated soils, even in desert biological soils crusts (Nicol *et al*., 2003; Poplanski *et al*., 2007; Angel *et al*., 2012; Conrad *et al*., 2012; Aschenbach *et al*., 2013).

The active archaeal community composition was less affected by the crop rotation than the resident community. However, several trends were identical to the ones already discussed for the resident community, e.g., the increase of the *Crenarchaeota*/*Thaumarchaeota* in the rotational fields, and the decrease of the methanogenic *Euryarchaeota* in the irrigated fields. The determination of rRNA/rDNA ratios showed that these values were indeed different between methanogenic and non-methanogenic phyla, which were higher or lower, respectively, in the rotational than in the irrigated fields. Such behavior, with increased rRNA/rDNA ratios in drained rice fields has been observed before (Watanabe *et al.*, 2007; **Chapter 3**, Breidenbach and Conrad, 2015). One should note that although "active" community members were defined by their ribosomal RNA, since the number of ribosomes is considered to reflect activity (e.g. Egert *et al*., 2011.), it remains unknown whether the microbes with increased numbers of ribosomes really expressed a higher level of activity. In fact, we believe that this is not necessarily the case, since increase of ribosomal RNA per cell can be interpreted as stress reaction in order to be prepared for potential better conditions (re-flooding) (**Chapter 3**, Breidenbach and Conrad, 2015). All together this leads to the conclusion that methanogenic archaeal lineages increased their ribosomal activity in order to be prepared for better conditions, while thaumarchaeotal lineages were stimulated by the crop rotation and increased their abundance

with active growth. These changes in the archaeal community were before the following season. Since sampling was done at the middle of each season, the microbes were exposed to upland conditions for nearly three months before the field was again flooded, possibly allowing the growth of aerobic *Crenarchaeota*/*Thaumarchaeota* during this time.

4.5.2 Impact of crop rotation on the bacterial community

The resident and active bacterial community was not dramatically impacted by the introduction of the upland maize cultivation. Only minor changes in relative abundance of several bacterial lineages were observed, mainly significant at the second period of maize cultivation during dry season 2013. These results were seen from the relative abundances of both bacterial sequences (16S rDNA and rRNA), most frequent core OTUs, and a selection of OTUs explaining most of the differences between the samples. Little effects on the bacterial communities have also been observed in an alfalfa-rice crop rotation (Lopes *et al*., 2014) and a winter wheat-rice- winter wheat-maize cropping system (Zhao *et al*., 2014). Furthermore, changes in the irrigation treatment in South Korean rice fields did not affect the overall bacterial community and impacted only the activity of some bacterial groups (Ahn *et al*., 2014). Since the texture of the Philippine rice field soil (silt loam) enables potential anaerobic microniches due to its high water holding capacity (**Chapter 3**, Breidenbach and Conrad, 2015) it is possible that anaerobic microorganisms potentially maintain their activity in these niches.

Only some bacterial lineages were affected by the crop rotation in the present study. For example, some OTUs of *Acidobacteria* exhibited higher relative abundance in the rotational fields while others, especially active *Acidobacteria* (assessed from rRNA sequences) were more numerous in the control rice fields. *Acidobacteria* are widely distributed and abundant soil microbes, which can adapt to low substrate availability and exhibit slow growth rates (Lee and Cho, 2009; Davis *et al*., 2005, 2011). Eichorst *et al*. (2011) showed that *Acidobacteria* are able to decompose complex carbon compounds like xylan, cellulose and pectin. Lopes *et al*. (2014) suggested *Acidobacteria* to play an important role in cycling of plant derived carbon in rice fields. High abundance of *Acidobacteria* was reported for ancient rice fields in China (Sheng *et al*., 2015). Conclusively, the relatively

high abundance of *Acidobacteria* in the Philippine fields may be the result of their versatile but central role in degradation of organic matter.

The second bacterial group of importance was the *Deltaproteobacteria*, namely *Anaeromyxobacter* and *Haliangiaceae* (*Haliangium*). The *Anaeromyxobacter* spp. are probably prominent iron reducers in the rice field soil (Ratering and Schnell, 2001; Treude *et al*., 2003). Drainage enabling increasing O_2 inflow in the soil may result in continuous regeneration of ferric iron thus supporting iron reducers within anoxic microniches, as suggested for South Korean rice fields (Ahn *et al*., 2014) and an unplanted and drained field on the present experimental site (**Chapter 3**, Breidenbach and Conrad, 2015). *Haliangium*, which is an aerobic bacterium (Fudou *et al*., 2002) was recently found to be enriched on rice roots in comparison to rhizospheric soil (Hernández *et al*., 2015).

Other *Deltaproteobacteria* were found to be in higher abundance in the control rice fields than the rotational fields, namely *Desulfobacterales* and *Syntrophobacterales*, which are known as sulfate reducers or syntrophic fermenting bacteria. For example, *Syntrophobacterales* may reduce sulfate and syntrophically interact with hydrogenotrophic methanogens (Kato and Watanabe, 2010). Therefore, it was hypothesized that they may be important for sulfate reduction and hydrogen production in rice fields under anoxic conditions (Itoh *et al*., 2013). Our observations support this hypothesis, since fields that were always managed as irrigated rice fields apparently provided a better environment for these bacteria than fields managed in rotation with upland crops.

A third group with relatively high abundance, both within the resident and the active communities of rotational and control fields, were members of the phylum *Chloroflexi*, e.g. *Anaerolineaceae.* Ahn *et al*., (2012) proposed *Chloroflexi* to be primary degraders of polysaccharides in anoxic rice field soil. Recently, increased abundance of *Chloroflexi* in flooded rice field in a rice-wheat cropping system was reported (Zhao *et al*., 2014). Accordingly, increased abundance of *Chloroflexi* was shown for rice fields during alfalfa-rice crop rotation (Lopes *et al*., 2014).

Lastly, *Firmicutes* were also present in the Philippine fields, albeit at relatively low abundance. These bacteria are suggested to tolerate drainage stress as they can form

endospores (Fernandez Scavino *et al*., 2013). Indeed we found an increased relative abundance of OTUs belonging to *Bacillales* in soil from the crop rotation compared to the control rice fields supporting this hypothesis.

4.5.3 Conclusion

The introduction of upland maize cultivation into an agricultural system dominated by irrigated rice cultivation and the concomitant change from mainly flooded to non-flooded conditions led to significant changes in the archaeal community. Methanogenic lineages decreased in abundance whereas non-methanogenic *Thaumarchaeota* relatively increased in the rotational fields. This change proved to be persistent over the next rotational cycles. On the other hand, the methanogenic archaeal community seemed to increase the number of ribosomes per cell, probably as a stress response reaction to the change in field management. Despite these changes it is noteworthy, that none of the methanogenic groups was lost from the soil but persisted despite adverse conditions (seen by the drastic decease of methane emission) of crop rotation. In contrast to the Archaea, only minor changes were observed in the bacterial community upon introduction of crop rotation, e.g., suppression of anaerobic syntrophic *Deltaproteobacteria* bacteria, enhancement of endospore-forming *Bacillales*, and diverse effects on members of *Acidobacteria* and *Chloroflexi*.

4.6 Supplemental material

Supplement Table 4.1 Soil variables in continuous rice fields (RR) and rice-maize crop rotation (RM) during dry and wet season 2012 and 2013.

Soil variable	Field type	2012		2013	
		Dry	*Wet*	*Dry*	*Wet*
Water content [g_{dw}^{-1}]	*RR*	42.8 ± 3.5	47.8 ± 3.5	64.5 ± 7.2	51.2 ± 3.6
	RM	34.3 ± 1.0	44.0 ± 5.0	51.5 ± 7.2	52.1 ± 4.2
pH	*RR*	6.5 ± 0.2	6.7 ± 0.0	6.7 ± 0.2	6.8 ± 0.1
	RM	6.1 ± 0.4	6.8 ± 0.0	5.5 ± 0.0	6.9 ± 0.1

Mean ± standard deviations of n=3 are shown.

Supplement Table 4.2 Number of archaeal 16S rDNA sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047229 for archaeal sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Name	*Season*	*Plant*	*Barcode*	*Accession*	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*	*Source*
RRF3	2012 dry	Rice	ATCGAT	SRS715487	60	0.85	104	20	[a]
RRF6	2012 dry	Rice	ATGCTA	SRS715488	126	0.95	220	37	[a]
RRF9	2012 dry	Rice	CACAGT	SRS715489	145	0.96	192	28	[a]
RMF4	2012 dry	Maize	CAGTCA	SRS715493	136	0.96	201	32	[a]
RMF7	2012 dry	Maize	CATGAC	SRS715494	139	0.96	197	34	[a]
RMF10	2012 dry	Maize	CGATAT	SRS715495	144	0.97	206	10	[a]
RRF3	2012 wet	Rice	ACGTAC		124	0.97	161	30	This study
RRF6	2012 wet	Rice	ACTGCA		112	0.93	169	30	This study
RRF9	2012 wet	Rice	AGAGTC		109	0.96	165	28	This study
RMF4	2012 wet	Rice	AGCTGA		116	0.95	151	33	This study
RMF7	2012 wet	Rice	AGTCAG		49	0.93	57	14	This study
RMF10	2012 wet	Rice	ATATCG		130	0.97	177	14	This study
RRF3	2013 dry	Rice	ACACGT		187	0.97	278	33	This study
RRF6	2013 dry	Rice	ACGTAC		192	0.98	266	34	This study
RRF9	2013 dry	Rice	ACTGCA		170	0.96	251	37	This study
RMF4	2013 dry	Maize	AGAGTC		119	0.97	164	5	This study
RMF7	2013 dry	Maize	AGCTGA		113	0.98	138	7	This study
RMF10	2013 dry	Maize	AGTCAG		63	0.94	79	4	This study
RRF3	2013 wet	Rice	ATATCG		195	0.96	377	10	This study
RRF6	2013 wet	Rice	ATCGAT		138	0.96	257	30	This study
RRF9	2013 wet	Rice	ATGCTA		220	0.97	295	39	This study
RMF4	2013 wet	Rice	CACAGT		214	0.98	273	24	This study
RMF7	2013 wet	Rice	CAGTCA		177	0.98	228	12	This study
RMF10	2013 wet	Rice	CATGAC		175	0.98	270	10	This study

[a] : Breidenbach and Conrad, 2015. Partial 16S rRNA primers: Archaea: Arch344F (5'-ACGGGGYGCAGCAGGCGCGA), Arch934br (5'-GTGCTCCCCCGCCAATTCCT). Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC)

Supplement Table 4.3 Number of archaeal16S rRNA sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047229 for archaeal sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Name	*Season*	*Plant*	*Barcode*	*Accession*	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*	*Source*
RRF3	2012 dry	Rice	ATCGAT	SRS715487	89	0.94	134	18	a
RRF6	2012 dry	Rice	ATGCTA	SRS715488	222	0.96	344	42	a
RRF9	2012 dry	Rice	CACAGT	SRS715489	182	0.97	293	28	a
RMF4	2012 dry	Maize	CAGTCA	SRS715490	128	0.96	177	15	a
RMF7	2012 dry	Maize	CATGAC	SRS715491	140	0.94	268	15	a
RMF10	2012 dry	Maize	CGATAT	SRS715492	160	0.98	253	11	a
RRF3	2012 wet	Rice	ACGTAC		108	0.93	145	23	This study
RRF6	2012 wet	Rice	ACTGCA		83	0.89	152	20	This study
RRF9	2012 wet	Rice	AGAGTC		77	0.91	121	14	This study
RMF4	2012 wet	Rice	AGCTGA		90	0.92	145	22	This study
RMF7	2012 wet	Rice	AGTCAG		55	0.89	106	10	This study
RMF10	2012 wet	Rice	ATATCG		130	0.97	191	15	This study
RRF3	2013 dry	Rice	CGATAT		183	0.99	272	4	This study
RRF6	2013 dry	Rice	CGCGCG		215	0.98	317	10	This study
RRF9	2013 dry	Rice	CGTATA		299	0.98	419	41	This study
RMF4	2013 dry	Maize	GACTAG		202	0.96	308	20	This study
RMF7	2013 dry	Maize	GAGATC		181	0.97	262	29	This study
RMF10	2013 dry	Maize	GATCGA		202	0.98	302	12	This study
RRF3	2013 wet	Rice	GTACAC		241	0.98	413	15	This study
RRF6	2013 wet	Rice	¶	-	-	-	-	-	-
RRF9	2013 wet	Rice	GTGTGT		215	0.99	266	9	This study
RMF4	2013 wet	Rice	TACGTA		237	0.98	358	32	This study
RMF7	2013 wet	Rice	TAGCAT		236	0.98	332	29	This study
RMF10	2013 wet	Rice	TATACG		198	0.98	327	17	This study

[a]: Breidenbach and Conrad, 2015. [¶]: sequencing failed. Partial 16S rRNA primers: Archaea: Arch344F (5'-ACGGGGYGCAGCAGGCGCGA), Arch934br (5'-GTGCTCCCCCGCCAATTCCT). Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC)

Supplement Table 4.4 Number of bacterial 16S rDNA sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047272 for bacterial sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Name	*Season*	*Plant*	*Barcode*	*Accession*	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*	*Source*
RRF3	2012 dry	Rice	ATCGAT	SRS715487	2342	0.83	4143	630	[a]
RRF6	2012 dry	Rice	ATGCTA	SRS715488	2098	0.85	3808	499	[a]
RRF9	2012 dry	Rice	CACAGT	SRS715489	1362	0.73	2806	525	[a]
RMF4	2012 dry	Maize	CAGTCA	SRS715490	1301	0.78	2519	470	[a]
RMF7	2012 dry	Maize	CATGAC	SRS715491	1256	0.77	2335	491	[a]
RMF10	2012 dry	Maize	CGATAT	SRS715492	1774	0.82	3392	416	[a]
RRF3	2012 wet	Rice	ACGTAC		2117	0.83	3806	563	This study
RRF6	2012 wet	Rice	ACTGCA		2144	0.82	3917	481	This study
RRF9	2012 wet	Rice	¶	-	-	-	-	-	-
RMF4	2012 wet	Rice	AGCTGA		2267	0.85	4093	531	This study
RMF7	2012 wet	Rice	AGTCAG		1277	0.76	2462	538	This study
RMF10	2012 wet	Rice	ATATCG		1481	0.81	2568	458	This study
RRF3	2013 dry	Rice	ACACGT		2742	0.86	4771	696	This study
RRF6	2013 dry	Rice	ACGTAC		1213	0.76	2474	408	This study
RRF9	2013 dry	Rice	ACTGCA		1102	0.73	2446	325	This study
RMF4	2013 dry	Maize	AGAGTC		2278	0.83	4125	587	This study
RMF7	2013 dry	Maize	AGCTGA		823	0.74	1885	158	This study
RMF10	2013 dry	Maize	AGTCAG		880	0.73	1962	341	This study
RRF3	2013 wet	Rice	ATATCG		1117	0.74	2539	332	This study
RRF6	2013 wet	Rice	ATCGAT		1104	0.71	2367	339	This study
RRF9	2013 wet	Rice	ATGCTA		1311	0.75	2674	330	This study
RMF4	2013 wet	Rice	CACAGT		996	0.72	2105	406	This study
RMF7	2013 wet	Rice	CAGTCA		898	0.71	2140	348	This study
RMF10	2013 wet	Rice	CATGAC		862	0.67	1933	520	This study

[a] : Breidenbach and Conrad, 2015. ¶: sequencing failed. Partial 16S rRNA primers: Bacteria: F515 (5'-GTGCCAGCNGCCGCGGTAA), R806 (5'-GGACTCVSGGGTATCTAAT). Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC).

Supplement Table 4.5 Number of bacterial 16S rRNA sequences before and after quality management, barcode, number of OTUs, coverage, Chao1 and inverted Simpson index of the environmental samples analyzed by 454-pyrosquencing. Raw data were deposited under the study accession numbers SRP047272 for bacterial sequences in the NCBI Sequence Read Archive (SRA). Sample from one field (A, B or C) was randomly chosen and analyzed as representative of the field.

Name	*Season*	*Plant*	*Barcode*	*Accession*	*No. OTU*	*Good´s coverage*	*Chao1*	*1/ Simpson*	*Source*
RRF3	2012 dry	Rice	ATCGAT	SRS715487	2829	0.86	4667	604	a
RRF6	2012 dry	Rice	ATGCTA	SRS715488	2636	0.88	4276	509	a
RRF9	2012 dry	Rice	CACAGT	SRS715489	2126	0.85	3507	587	a
RMF4	2012 dry	Maize	CAGTCA	SRS715490	2138	0.87	3558	253	a
RMF7	2012 dry	Maize	CATGAC	SRS715491	1966	0.83	3493	308	a
RMF10	2012 dry	Maize	CGATAT	SRS715492	1505	0.83	2482	307	a
RRF3	2012 wet	Rice	ACGTAC		3500	0.89	6037	652	This study
RRF6	2012 wet	Rice	ACTGCA		3160	0.85	5581	725	This study
RRF9	2012 wet	Rice	¶	-	-	-	-	-	-
RMF4	2012 wet	Rice	AGCTGA		2774	0.86	4837	578	This study
RMF7	2012 wet	Rice	AGTCAG		2850	0.86	5246	517	This study
RMF10	2012 wet	Rice	ATATCG		3199	0.86	5895	500	This study
RRF3	2013 dry	Rice	CGATAT		771	0.72	1858	209	This study
RRF6	2013 dry	Rice	CGCGCG		1895	0.85	3222	386	This study
RRF9	2013 dry	Rice	CGTATA		1440	0.75	2669	585	This study
RMF4	2013 dry	Maize	GACTAG		1844	0.82	3196	464	This study
RMF7	2013 dry	Maize	GAGATC		2226	0.88	3653	230	This study
RMF10	2013 dry	Maize	GATCGA		1820	0.85	2997	386	This study
RRF3	2013 wet	Rice	GTACAC		2347	0.88	3957	377	This study
RRF6	2013 wet	Rice	GTCACA		899	0.72	1843	303	This study
RRF9	2013 wet	Rice	GTGTGT		1266	0.78	2574	368	This study
RMF4	2013 wet	Rice	TACGTA		802	0.71	1713	244	This study
RMF7	2013 wet	Rice	TAGCAT		930	0.73	1944	206	This study
RMF10	2013 wet	Rice	TATACG		779	0.69	1643	295	This study

[a] : Breidenbach and Conrad, 2015. ¶: sequencing failed. Partial 16S rRNA primers: Bacteria: F515 (5'-GTGCCAGCNGCCGCGGTAA), R806 (5'-GGACTCVSGGGTATCTAAT). Adaptor primes: forward (5'-GATGGCCATTACGGCC), reverse (5'-GGTGGCCGAGGCGGCC).

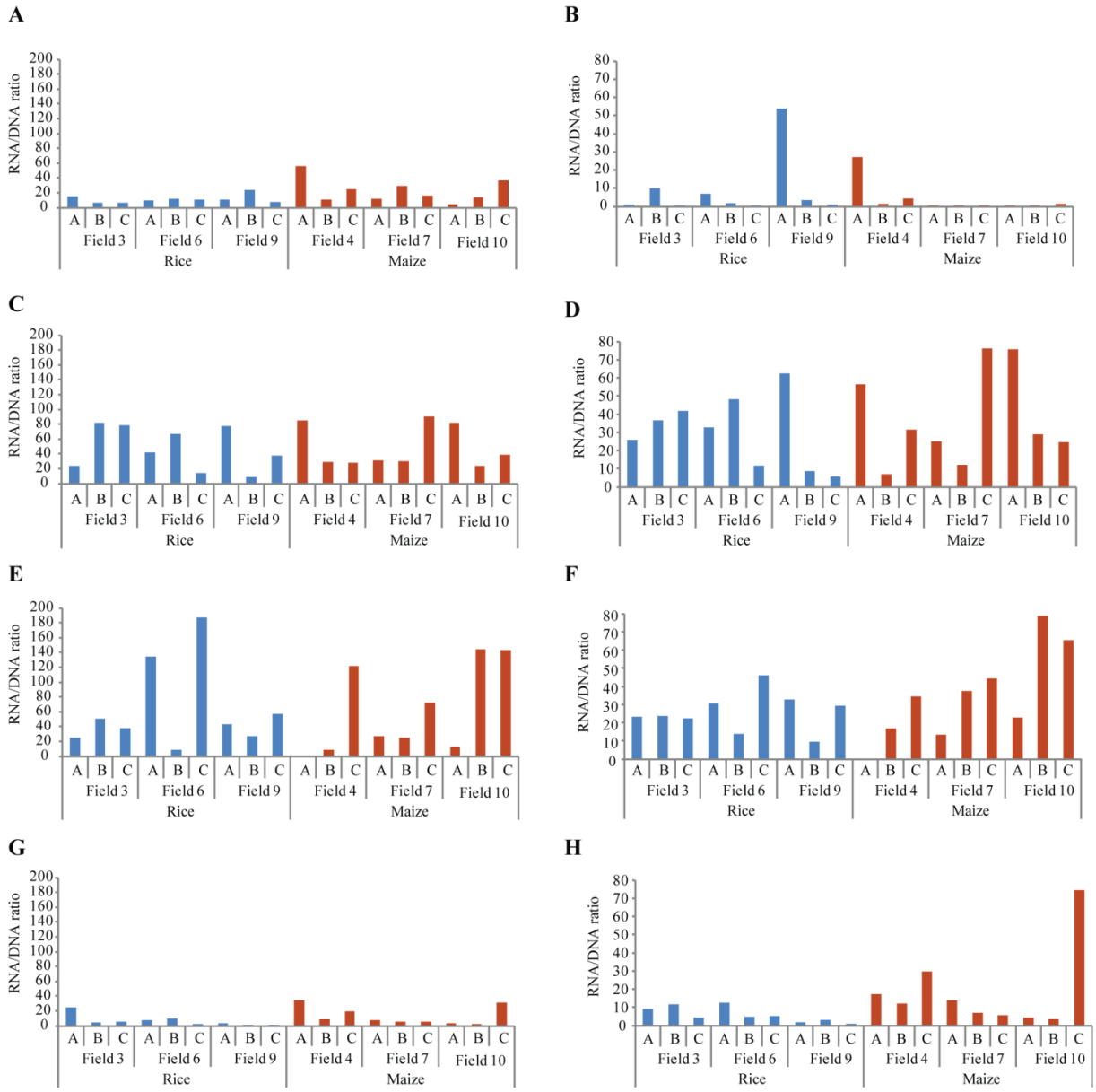
Supplement Table 4.6 Relative number of sequences (%) represented by core (C, bold) and unique (U, light) OTUs of the bacterial and the top 50 OTUs of the archaeal community in continuous rice fields (RR) and rice-maize crop rotation (RM).

Target gene	*2012*								*2013*							
	Dry				*Wet*				*Dry*				*Wet*			
	RR		*RM*		*RR*		*RM*		*RR*		*RM*		*RR*		*RM*	
Bacteria																
	C	U	**C**	*U*	**C**	*U*	**C**	*U*	**C**	*U*	**C**	*U*	**C**	*U*	**C**	*U*
16S rDNA	**49.1**	5.6	**54.7**	4.2	**33.2**	14.2	**51.5**	4.7	**53.7**	6.5	**57.4**	5.2	**56.8**	8.5	**57.2**	7.0
16S rRNA	**49.5**	2.5	**66.8**	5.4	**76.0**	4.3	**66.5**	5.7	**70.1**	4.8	**65.9**	6.1	**74.2**	4.2	**68.1**	4.5
Archaea	***Top 50 OTUs***															
16S rDNA	76.2		76.6		78.4		77.6		75.5		87.5		63.7		81.1	
16S rRNA	79.4		80.9		83.1		77.9		76.9		76.5		84.1		77.5	

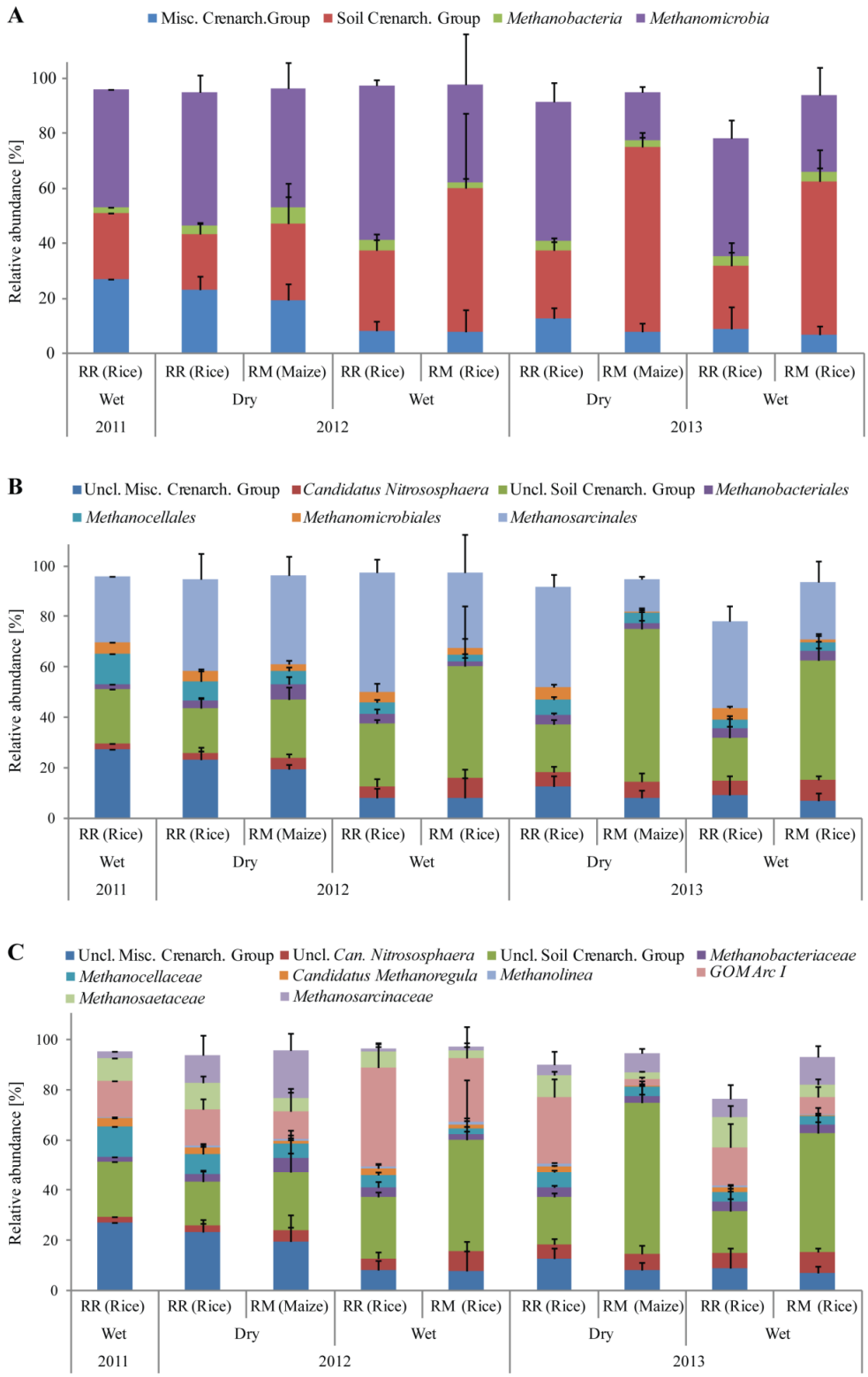
Supplement Table 4.7 Taxonomic assignment of archaeal OTUs based on 16S rDNA and 16S rRNA. Assignments down to the lowest taxonomic level are shown. Taxonomic level and rank are given along with taxon, number of detected OTUs and OTU name.

			16S rDNA		*16S rRNA*	
Tax. level	***rankID***	***Taxon***	***No. OTUs***	***OTU Name***	***No. OTUs***	***OTU Name***
1	*0.1*	*Archaea*				
2	*0.1.2*	*Crenarchaeota*	1	15	1	3
3	*0.1.2.8*	*Misc. Crenarchaeotic Group*	7	6, 12, 19, 33, 48, 266, 349	5	13, 20, 21, 38, 516
3	*0.1.2.9*	*Soil Crenarchaeotic Group*	8	1, 2, 8, 13, 20, 123, 226, 510	7	1, 2, 5, 9, 12, 140, 428
4	*0.1.2.9.1*	*Candidatus Nitrososphaera*	3	4, 103, 370	3	4, 34, 219
3	*0.1.2.12*	*Thermoprotei*			1	600
2	*0.1.3*	*Euryarchaeota*				
6	*0.1.3.3.1.1.1*	*Methanobacterium*	2	16, 54	2	11, 48
6	*0.1.3.5.4.1.1*	*Methanocella*	3	17, 24, 26	3	14, 23, 25
4	*0.1.3.5.5*	*Methanomicrobiales*			1	43
5	*0.1.3.5.5.1*	*Candidatus Methanoregula*	2	25, 27	1	33
5	*0.1.3.5.5.4*	*Methanolinea*	1	21	1	26
4	*0.1.3.5.6*	*Methanosarcinales*				
5	*0.1.3.5.6.3*	*GOM Arc I*	10	10, 14, 18, 22, 29, 30, 229, 283, 391, 554	6	10, 15, 17, 19, 29, 40
6	*0.1.3.5.6.5.1*	*Methanosaeta*	6	11, 28, 31, 32, 40, 56	9	6, 18, 24, 27, 35, 36, 182, 288, 544
6	*0.1.3.5.6.6.5*	*Methanolobus*			1	46
6	*0.1.3.5.6.6.8*	*Methanosarcina*	4	5, 38, 52, 359	6	7, 39, 195, 226, 295, 621
2	*0.1.6*	*unclassified*	3	3, 7, 9	3	8, 16, 143

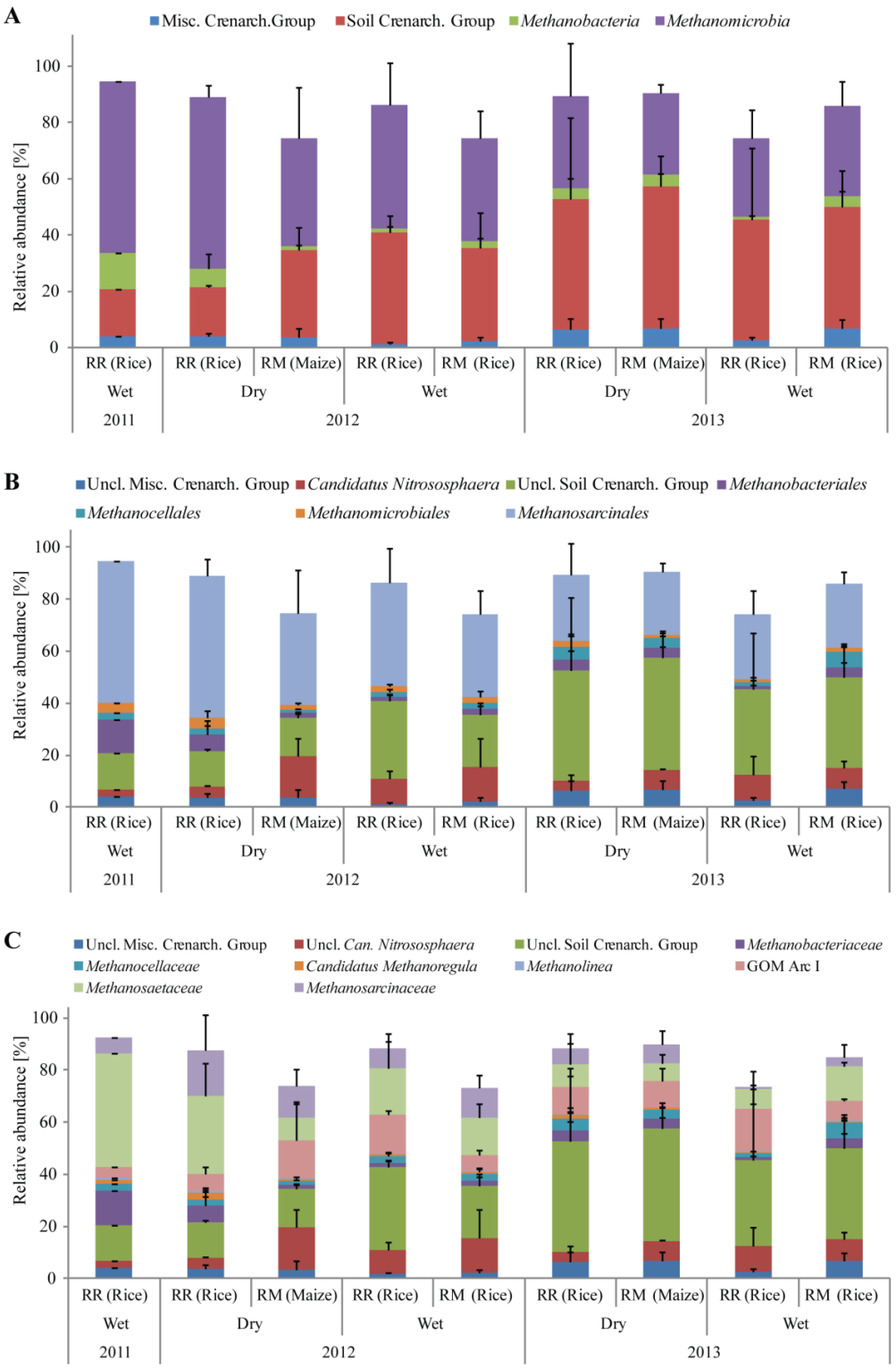
Tax. Level: taxonomic level; rankID: taxonomic rank; No. OTUs: number of detected OTUs.



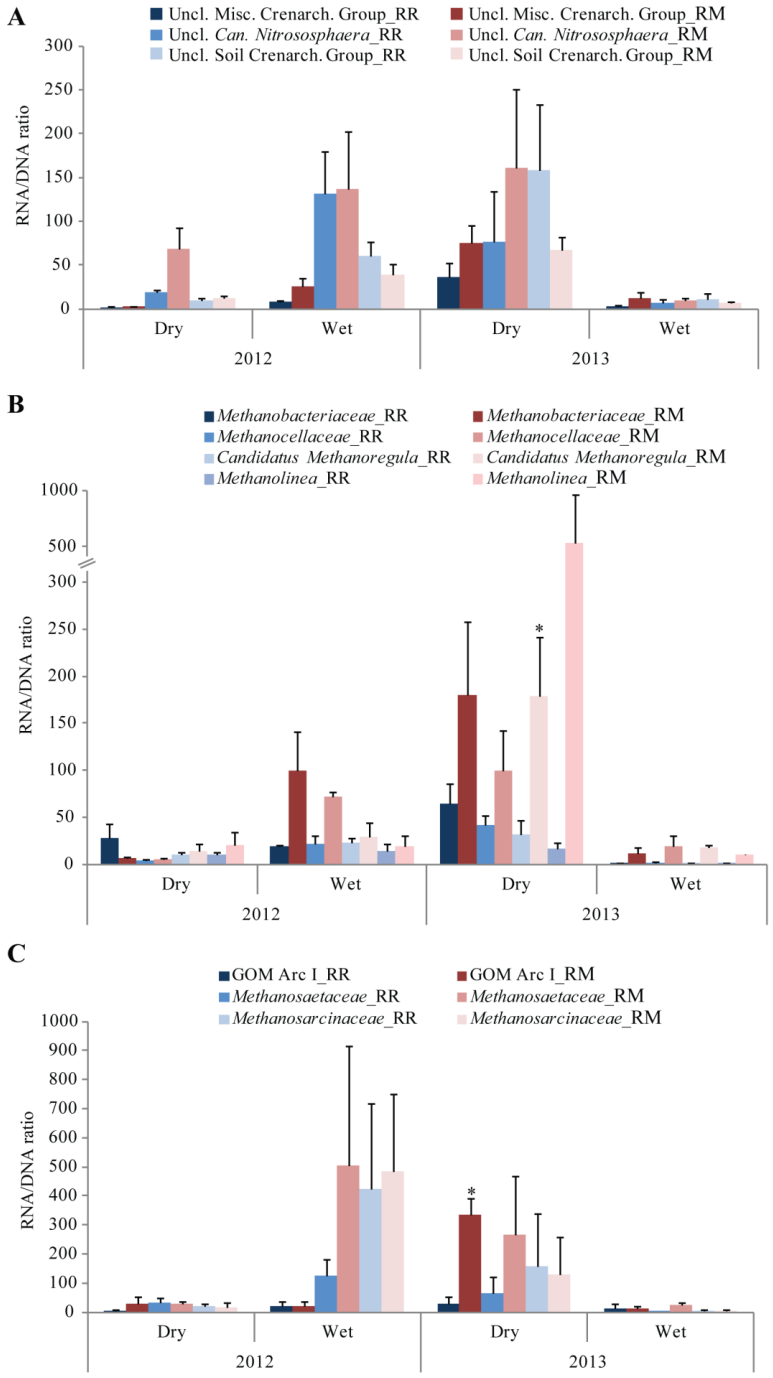
Supplement Figure 4.1 Ratio of ribosomal 16S rRNA and rDNA copy numbers quantified by qPCR. Archaeal (**A, C, E, G**) and bacterial (**B, D, F, H**) ratios are shown for each replicate during dry and wet season 2012 (**A, B; C, D**) and 2013 (**E, F; G, H**) in the fields with control rice cultivation (RR; blue) and the flooded rice-upland maize crop rotational fields (RM, red).



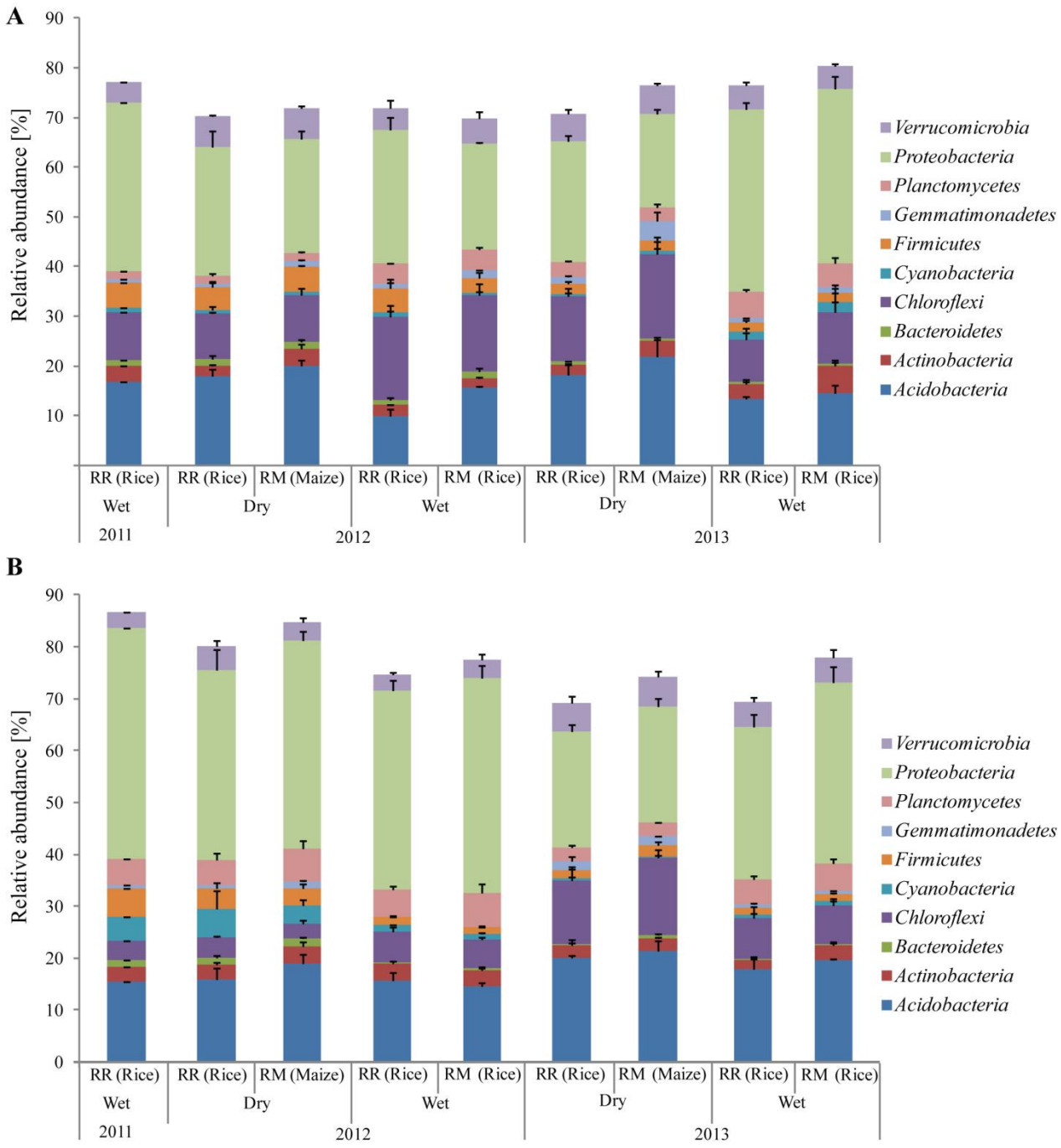
Supplement Figure 4.2 Relative abundance of archaeal sequences based on 454 pyrosequencing. 16S rDNA sequences obtained in control rice fields (RR) and rice-upland maize crop rotation (RM) are shown on class (**A**), order (**B**) and family level (**C**). Bars represent standard deviations of n=3.



Supplement Figure 4.3 Relative abundance of archaeal sequences based on 454 pyrosequencing. 16S rRNA sequences obtained in control rice fields (RR) and rice-upland maize crop rotation (RM) are shown on class (**A**), order (**B**) and family level (**C**). Bars represent standard deviations of n=3.



Supplement Figure 4.4 Ratio of ribosomal 16S rRNA/rDNA copy numbers multiplied by the relative sequence abundance of archaeal classes. Ratios of *Thaumarchaeota* (**A**), *Methanobacteria* (**B**) and *Methanomicrobia* (**C**) on family level derived ratios are shown for dry and wet season 2012 and 2013 in the fields with control rice cultivation (RR; blue) and the flooded rice-upland maize crop rotational fields (RM, red) are displayed. Columns represent mean and bars standard errors of n=3. Asterisk represents statistically significant differences at one particular season (ANOVA, $P < 0.05$).



Supplement Figure 4.5 Relative abundance of bacterial sequences based on 454 pyrosequencing. 16S rDNA (**A**) and 16S rRNA (**B**) sequences obtained in control rice fields (RR) and rice-upland maize crop rotation (RM) are shown on order level. Bars represent standard deviations of n=3.

4.7 Acknowledgement

This work has been funded as part of the ICON consortium (BR2238/9-1). We thank the German Research Foundation (DFG) for funding (FOR 1701, 'Introducing Non-Flooded Crops in Rice-Dominated Landscapes: Impacts on Carbon, Nitrogen and Water Cycles [ICON]'. We are thankful to Franziska B. Brandt for valuable comments on the manuscript. Furthermore, we thank the International Rice Research Institute and Reiner Wassmann for providing research space and support during sample collection. We thank Mary Louise Mendoza, Eugene Aquino and Jerico Stefan Bigornia for sample collection. We thank Peter Frenzel for valuable comments on the study. We thank the Max-Planck-Genome-Center in Cologne for access to the sequencing facility and support.

4.8 References

Abdi, H., and Williams, L. J. (2010). Principal component analysis. *WIREs Comp. Stat.* 2, 433-459.

Ahn, J. H., Choi, M. Y., Kim, B. Y., Lee, J. S., Song, J., Kim, G. Y., and Weon, H. Y. (2014). Effects of water-saving irrigation on emissions of greenhouse gases and prokaryotic communities in rice paddy soil. *Microb. Ecol.* 68, 271-283. doi: 10.1007/s00248-014-0371-z.

Ahn, J. H., Song, J., Kim, B. Y., Kim, M. S., Joa, J. H., and Weon, H. Y. (2012). Characterization of the bacterial and archaeal communities in rice field soils subjected to long-term fertilization practices. *J. Microbiol.* 50, 754-765.

Angel, R., Claus, P., and Conrad, R. (2012). Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *ISME J.* 6, 847-862.

Asakawa, S., and Hayano, K. (1995). Population of methanogenic bacteria in paddy field soil under double cropping conditions (rice-wheat). *Biol. Fertil. Soils* 20, 113-117.

Aschenbach, K., Conrad, R., Řeháková, K., Doležal, J., Janatková, K., and Angel, R. (2013). Methanogens at the top of the world: occurrence and potential activity of methanogens in newly deglaciated soils in high-altitude cold deserts in the Western Himalayas. *Front. Microbio*. 4, 359. doi:10.3389/fmicb.2013.00359.

Bates, S. T., Cropsey, G. W., Caporaso, J. G., Knight, R., and Fierer, N. (2011). Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol*. 77, 1309-1314. doi: 10.1128/AEM.02257-10.

Bertomeu, M. (2012). Growth and yield of maize and timber trees in smallholder agroforestry systems in Claveria, northern Mindanao, Philippines. *Agrofor. Syst.* 84, 73-87.

Bouman, B. A. M., Peng, S., Castaneda, A. R., and Visperas, R. M. (2005). Yield and water use of irrigated tropical aerobic rice systems. *Agric. Water Manag,* 74, 87-105.

Breidenbach, B., and Conrad, R. (2015). Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Front. Microbiol*. 5, 752. doi: 10.3389/fmicb.2014.00752

Burggraf, S., Huber, H., and Stetter, K.O. (1997). Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *Int. J. Syst. Evol. Microbiol*. 47, 657-660.

Bürgmann, H., Pesaro, M., Widmer, F., and Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. *J. Microbiol. Meth*. 45, 7-20.

Casamayor, E. O., Massana, R., Benlloch, S., Øvreås, L., Díez, B., Goddard, V. J., *et al*. (2002). Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. *Environ. Microbiol*. 4, 338-348.

Conrad, R. (2007). Microbial ecology of methanogens and methanotrophs. *Adv. Agron.* 96, 1-63.

Conrad, R., and Frenzel, P. (2002). "Flooded soils". in: *Encycl. Environ. Microbio.*, ed. G. Britton, John Wiley and Sons, New York, USA, 1316-1333. doi:10.1002/0471263397.env034.

Conrad, R., Klose, M., Lu, Y., and Chidthaisong, A. (2012). Methanogenic pathway and archaeal communities in three different anoxic soils amended with rice straw and maize straw. *Front Microbiol* 3, 4. doi:10.3389/fmicb.2012.00004.

Davis, K. E., Joseph, S. J., and Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl. Environ. Microbiol.* 71, 826-834.

Davis, K. E., Sangwan, P., and Janssen, P. H. (2011). *Acidobacteria, Rubrobacteridae* and *Chloroflexi* are abundant among very slow-growing and mini-colony-forming soil bacteria. *Environ. Microbiol*. 13, 798-805.

Deng, Y., Cui, X., Hernández, M., and Dumont, M. G. (2014). Microbial diversity in hummock and hollow soils of three wetlands on the Qinghai-Tibetan plateau revealed by 16S rRNA pyrosequencing. *PloS one*, 9, E103115 doi:10.1371/journal.pone.0103115.

Dutaur, L., and Verchot, L. V. (2007). A global inventory of the soil CH_4 sink. *Global Biogeochem. Cycles* 21, GB4013, doi:10.1029/2006GB002734.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996-998.

Egert, M., Schmidt, I., Höhne, H. M., Lachnit, T., Schmitz, R. A., and Breves, R. (2011). rRNA-based profiling of bacteria in the axilla of healthy males suggests right–left asymmetry in bacterial activity. *FEMS Microbiol. Ecol.* 77, 146-153.

Eichorst, S. A., Kuske, C. R., and Schmidt, T. M. (2011). Influence of plant polymers on the distribution and cultivation of bacteria in the phylum *Acidobacteria*. *Appl. Environ. Microbiol.* 77, 586-596.

Eusufzai, M. K., Tokida, T., Okada, M., Sugiyama, S., Liu, G. C., Nakajima, M., and Sameshima, R. (2010). Methane emission from rice fields as affected by land use change. *Agric. Ecosyst. Environ.* 139, 742-748.

Fernandez Scavino, A., Ji, Y., Pump, J., Klose, M., Claus, P., and Conrad, R. (2013). Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated rice fields. *Environ. Microbiol*. 15, 2588-2602.

Fudou, R., Jojima, Y., Iizuka, T., and Yamanaka, S. (2002). *Haliangium ochraceum* gen. nov., sp. nov. and *Haliangium tepidum* sp. nov.: novel moderately halophilic myxobacteria isolated from coastal saline environments. *J. Gen. Appl. Microbiol*. 48, 109-116.

Großkopf, R., Janssen, P. H., and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64, 960-969.

Haichar, Z. F., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., *et al*. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME J*. 2, 1221-1230.

Haroon, M. F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., *et al*. (2013). Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* 500, 567-570.

Heinz, E., Kraft, P., Buchen, C., Frede, H. G., Aquino, E., and Breuer, L. (2013). Set up of an automatic water quality sampling system in irrigation agriculture. *Sensors* 14, 212-228.

Hernández, M., Dumont, M. G., Yuan, Q., and Conrad, R. (2015). Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. *Appl. Environ. Microbiol.* doi:10.1128/AEM.03209-14.

Itoh, H., Ishii, S., Shiratori, Y., Oshima, K., Otsuka, S., Hattori, M., and Senoo, K. (2013). Seasonal transition of active bacterial and archaeal communities in relation to water management in paddy soils. *Microbes Environ*. 28, 370-380.

Jäckel, U., Schnell, S., and Conrad, R. (2001). Effect of moisture, texture and aggregate size of paddy soil on production and consumption of CH_4. *Soil. Biol. Biochem.* 33, 965-971.

Kato, S., and Watanabe, K. (2010). Ecological and evolutionary interactions in syntrophic methanogenic consortia. *Microbes Environ*. 25, 145-151.

Kenmore, Z. F., and Flinn, J. C. (1987). An ethnohistory of an upland area: Claveria, Misamis Oriental. International Rice Research Institute (IRRI), Manila, Philippines.

Klein, D. A., Frederick, B. A., Biondini, M., and Trlica, M. J. (1988). Rhizosphere microorganism effects on soluble amino acids, sugars and organic acids in the root zone of *Agropyron cristatum*, *A. smithii* and *Bouteloua gracilis*. *Plant Soil* 110, 19-25.

Krüger, M., Frenzel, P., and Conrad, R. (2001). Microbial processes influencing methane emission from rice fields. *Global Change Biol*. 7, 49-63.

Krüger, M., Frenzel, P., Kemnitz, D. and Conrad, R. (2005). Activity, structure and dynamics of the methanogenic archaeal community in a flooded Italian rice field. *FEMS Microbiol. Ecol*. 51, 323-33.

Lee, S. H., and Cho, J. C. (2009). Distribution patterns of the members of phylum acidobacteria in global soil samples. *J. Microbiol. Biotech*. 19, 1281-1287.

Liu, D., Ishikawa, H., Nishida, M., Tsuchiya, K., Takahashi, T., Kimura, M., and Asakawa, S. (2015). Effect of paddy-upland rotation on methanogenic archaeal community structure in paddy field soil. *Microb. Ecol*. 69, 160-168.

Lopes, A. R., Manaia, C. M., and Nunes, O. C. (2014). Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. *FEMS Microbiol. Ecol.* 87, 650-663.

Lu, Y., and Conrad, R. (2005). In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309, 1088-1090.

Ma, K., and Lu, Y. (2011). Regulation of microbial methane production and oxidation by intermittent drainage in rice field soil. *FEMS Microbiol. Ecol.* 75, 446-456.

Maclean, J. L., Dawe, D. C., Hardy, B., and Hettel, G. P. (eds) (2002). "Rice almanac: source book for the most important economic activity on earth, 3rd edn" CABI Publishing, Wallingford, United Kingdom.

Marschner, P., Crowley, D., and Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil*, 261, 199-208.

Marschner, P., Yang, C. H., Lieberei, R., and Crowley, D. E. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol. Biochem.* 33, 1437-1445.

Martinez, R.J., Mills, H.J., Story, S., and Sobecky, P.A. (2006). Prokaryotic diversity and metabolically active microbial populations in sediments from an active mud volcano in the Gulf of Mexico. *Environ. Microbiol.* 8, 1783-1796.

Megonigal, J. P., and Guenther, A. B. (2008). Methane emissions from upland forest soils and vegetation. *Tree Physiol.* 28, 491-498.

Mills, H. J., Martinez, R. J., Story, S., and Sobecky, P. A. (2005). Characterization of microbial community structure in Gulf of Mexico gas hydrates: comparative analysis of DNA- and RNA-derived clone libraries. *Appl. Environ. Microbiol.* 71, 3235-3247.

Nicol, G. W., Glover, L. A., and Prosser, J. I. (2003). The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. *Environ. Microbiol.* 5, 152-162.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382-395.

Oksanen, J., Blanchet, G. F., Kindt, R., Legendre, R., Minchin, P. R., O'Hara, R. B., *et al*. (2012). vegan: Community Ecology Package ver. 2.0-5. Available online at: http://cran.r-project.org/web/packages/vegan/index.html

Pester, M., Schleper, C., and Wagner, M. (2011). The *Thaumarchaeota*: an emerging view of their phylogeny and ecophysiology. *Curr. Opin. Microbiol.* 14, 300-306.

Ponnamperuma, F.N. (1972). The chemistry of submerged soils. *Adv.Agron*. 24, 29-96.

Poplawski, A. B., Martensson, L., Wartiainen, I., and Rasmussen, U. (2007). Archaeal diversity and community structure in a Swedish barley field: specificity of the Ek510r/ (EURY498) 16S rDNA primer. *J. Microbiol. Methods* 69, 161-173.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188-7196

Pump, J., Pratscher, J., and Conrad, R. (2014). Colonization of rice roots with methanogenic archaea controls photosynthesis-derived CH_4 emission. *Environ. Microbiol. Rep.* doi:10.1111/1462-2920.12675

R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, Available online at: http://www.R-project.org/.

Ratering, S., and Schnell, S. (2001). Nitrate-dependent iron (II) oxidation in paddy soil. *Environ. Microbiol.* 3, 100-109.

Ratering S., and Conrad, R. (1998). Effects of short-term drainage and aeration on the production of methane in submerged rice soil. *Glob. Change Biol.* 4, 397-407.

Rui, J., Peng, J., and Lu, Y. (2009). Succession of bacterial populations during plant residue decomposition in rice field soil. *Appl. Environ. Microbiol.* 75, 4879-4886.

Schloss, P. D., Gevers, D., and Westcott, S. L. (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS ONE* 6: e27310. doi:10.1371/journal.pone.0027310

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., *et al*. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537-7541.

Sheng, R., Qin, H., O'Donnell, A. G., Huang, S., Wu, J., and Wei, W. (2015). Bacterial succession in paddy soils derived from different parent materials. *J. Soil. Sediment*. 15, 982-992.

Shrestha, P. M., Noll, M., and Liesack, W. (2007). Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. *Environ. Microbiol.* 9, 2464-2474.

Soussana, J. F., Allard, V., Pilegaard, K., Ambus, P., Amman, C., Campbell, C., *et al*. (2007). Full accounting of the greenhouse gas (CO_2, N_2O, CH_4) budget of nine European grassland sites. *Agric. Ecosyst. Environ.* 121, 121-134.

Stieglmeier, M., Klingl, A., Alves, R. J., Simon, K. M. R., Melcher, M., Leisch, N., and Schleper, C. (2014). *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and

mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota. Int. J. Syst. Evol. Microbiol*, 64, 2738-2752.

Stubner, S. (2002). Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen™ detection. *J. Microbiol. Meth*. 50, 155-164.

Timsina, J., Jat, M. L., and Majumdar, K. (2010). Rice-maize systems of South Asia: current status, future prospects and research priorities for nutrient management. *Plant Soil* 335, 65-82.

Treude, N., Rosencrantz, D., Liesack, W., and Schnell, S. (2003). Strain FAc12, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales*. *FEMS Microbiol. Ecol.* 44, 261-269.

Tuong, T. P., Bouman, B. A. M., and Mortimer, M. (2005). More rice, less water-integrated approaches for increasing water productivity in irrigated rice-based systems in Asia. *Plant Prod. Sci.* 8, 231-241.

Van Nguyen, N., and Ferrero, A. (2006). Meeting the challenges of global rice production. *Paddy Water Environ*. 4, 1-9. doi: 10.1007/s10333-005-0031-5.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol*. 73, 5261-5267.

Warnes, G. R. (2011) Gplots: Various R programming tools for plotting data. http://cran.r-project.org/web/packages/gplots/index.html

Wassmann, R., Buendia, L. V., Lantin, R. S., Bueno, C. S., Lubigan, L. A., Umali, A., *et al* (2000a). Mechanisms of crop management impact on methane emissions from rice fields in Los Banos, Philippines. *Nutr Cycl Agroecosyst* 58, 107-119. doi:10.1023/A:1009838401699

Wassmann, R., Lantin, R. S., Neue, H. U., Buendia, L. V., Corton, T. M., and Lu, Y. (2000b) Characterization of methane emissions from rice fields in Asia. III. Mitigation options and future research needs. *Nutr Cycl Agroecosyst* 58, 23-36. doi:10.1023/A:1009874014903

Watanabe, T., Hosen, Y., Agbisit, R., Llorca, L., Katayanagi, N., Asakawa, S., and Kimura, M. (2013). Changes in community structure of methanogenic archaea brought about by water-saving practice in paddy field soil. *Soil Biol. Biochem*. 58, 235-243.

Watanabe, T., Kimura, M., and Asakawa, S. (2006) Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). *Soil Biol. Biochem*. 38, 1264-1274.

Watanabe, T., Kimura, M., and Asakawa, S. (2007). Dynamics of methanogenic archaeal communities based on rRNA analysis and their relation to methanogenic activity in Japanese paddy field soils. *Soil Biol. Biochem*. 39, 2877-2887.

Watanabe, T., Kimura, M. and Asakawa, S. (2009). Distinct members of a stable methanogenic archaeal community transcribe *mcrA* genes under flooded and drained conditions in Japanese paddy field soil. *Soil Biol. Biochem*. 41, 276-285.

Watanabe, T., Wang, G., Lee, C. G., Murase, J., Asakawa, S., and Kimura, M. (2011). Assimilation of glucose-derived carbon into methanogenic archaea in soil under unflooded condition. *Appl. Soil Ecol*. 48, 201-209.

Weller, S., Kraus, D., Ayag, K. R. P., Wassmann, R., Alberto, M. C. R., Butterbach-Bahl, K., and Kiese, R. (2015a). Methane and nitrous oxide emissions from rice and maize production in diversified rice cropping systems. *Nutr. Cycl. Agroecosyst*. 101, 37-53.

Weller, S., Kraus, D., Racela, H. S., Wassmann, R., Butterbach-Bahl K., and Kiese, R. (2015b). Two-year automated measurement of methane and nitrous oxide emissions from paddy rice based rotation systems in the Philippines CH_4 and N_2O measurements in rice rotation systems. (*In preparation*)

Yagi, K., Tsuruta, H., Kanda, K., and Minami, K. (1996). Effect of water management on methane emission from a Japanese rice paddy field: auto-mated methane monitoring. *Glob. Biogeochem. Cycles* 10, 255-267. doi:10.1029/96GB00517.

Zhang, G., Ji, Y., Ma, J., Xu, H., Cai, Z., and Yagi, K. (2012). Intermittent irrigation changes production, oxidation, and emission of CH_4 in paddy fields determined with stable carbon isotope technique. *Soil. Biol. Biochem.* 52, 108-116.

Zhao, J., Zhang, R., Xue, C., Xun, W., Sun, L., Xu, Y., and Shen, Q. (2014). Pyrosequencing reveals contrasting soil bacterial diversity and community structure of two main winter wheat cropping systems in China. *Microb. Ecol*. 67, 443-453.

Zhou, L., Wang, Y., Long, X. E., Guo, J., and Zhu, G. (2014). High abundance and diversity of nitrite-dependent anaerobic methane-oxidizing bacteria in a paddy field profile. *FEMS Microbiol. Ecol.* 360, 33-41.

Zhu, W., Lu, H., Hill, J., Guo, X., Wang, H., and Wu, W. (2014). ^{13}C pulse-chase labeling comparative assessment of the active methanogenic archaeal community composition in the transgenic and nontransgenic parental rice rhizospheres *FEMS Microbiol. Ecol.* 87, 746-756.

Zinger, L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M., Huse, S. M., Welch, D. B., *et al*. (2011). Global patterns of bacterial beta-diversity in seafloor and sea-water ecosystems. *PLoS ONE* 6:e24570. doi:10.1371/ journal.pone.0024570.

Chapter 5

Discussion and concluding remarks

Rice is a major staple food and its demand is anticipated to increase along with the increasing world population. Rice agriculture provides around 10% of the global atmospheric methane budget. The biogeochemistry of rice fields as wetlands and their inhabiting microbial community has been studied extensively, however numerous open questions still remain unclear.

In this thesis the impact of the rice plant on the microbial community living in the soil was investigated in a greenhouse experiment comparing planted and unplanted rice field soil (**Chapter 2**). Further, dynamics in the microbial community during the rice plant growth stages were elucidated by studying a time series **(Chapter 2)**. The microbial community composition and abundance was assessed using several molecular techniques (T-RFLP, 454 pyrosequencing, qPCR) targeting the ribosomal 16S rRNA gene (16S rDNA) allowing to monitor the resident microbes. The issue of potential impacts of rice plant growth stages on the microbial community was further tackled as part of a field study embedded in the multidisciplinary research project ”Introduction of non-flooded crops in rice-dominated landscapes and its impact on carbon, nitrogen and water cycles (ICON)” conducted at experimental fields of the International Rice Research Institute (IRRI) in the Philippines (**Chapter 3**). Additionally, in this part of the thesis the active microbial community was assessed targeting the ribosomal RNA (16S rRNA).

The second part of this thesis concentrated on consequences of crop rotational systems on the microbial community in the rice field soil. As a part of the ICON project we studied the impact of the introduction of an upland plant (maize) and concomitant drainage on the rice soil microbial community. We used several molecular techniques targeting the ribosomal 16S rDNA and 16S rRNA allowing to examine the resident and active community in abundance and composition. In **Chapter 3** the immediate reaction of the microbial community to changes in field management was studied. Subsequent, we followed the crop

rotation over two years by comparing the rotational fields to a field solely managed as flooded rice field. The long term effects of the crop rotation were reported in **Chapter 4**.

5.1 Microbial communities in the rice rhizosphere

Rhizodeposition, a process excreting organic and inorganic compounds, is a mechanism regularly used by plants (among others, e.g. nutrient uptake) to influence the microbial community in the soil surrounding the roots. The compounds, called rhizodeposits, are excreted via the plant root. For rice plants it was shown that the quantity and quality of these rhizodeposits change with the plant growth stage (Aulakh *et al*., 2001). Further, rhizodeposits are the major carbon input into the soil besides plant residues and soil organic matter (Kimura *et al*., 2004). Therefore, a pot experiment was conducted to investigate whether the microbial community in the rhizosphere differs from the community in unplanted soil and whether dynamics within the rice plant growth stages occur (**Chapter 2**). Little impact of plant growth stage was observed on the resident archaeal and bacterial community. More distinct differences were shown between the microbial communities in the rhizosphere and in the unplanted bulk soil suggesting a plant effect. On field scale similar patterns were observed confirming previous results of the greenhouse experiment (**Chapter 3**). Only a few microbial lineages differed between the rice growth stages. In contrast, stronger variations were found between the planted and the unplanted fields. However, these were mainly accounted to the difference in water management as the planted fields were flooded and the unplanted fields drained. In contrast, during the greenhouse experiment in **Chapter 2** both planted and unplanted pots were treated equally, as they were flooded throughout the experiment. Together, these results may indicate that the overall microbial community in rice field soil is only minor affected by the rice plant. However, focusing on specific microbial lineages we were able to detect differences (**Chapter 2, 3**). Further, stable isotope tracer studies were able to identify the bacterial and archaeal lineages assimilating carbon compounds derived from the rice plant in the rhizosphere or in the rhizoplane (root surface) (Lu and Conrad, 2006; Zhu *et al*., 2014; Hernández *et al*., 2015). Additional, distinct microbial communities in the endosphere (root interior), the rhizoplane and the rhizosphere of rice were reported (Edwards *et al*., 2015). Therefore, the distinct sampling seems to be of high importance since different microbial communities were identified inhabiting specific locations next to the plant, on the plant or even inside the plant. In this thesis we focused on the soil attached to the rice roots (rhizosphere; **Chapter 2**) and in the

vicinity of the plant (**Chapter 3**) enabling the detection of dynamics in the microbial community.

The observed stability of the overall microbial community may be a consequence of the adaption to the rice field ecosystem. The rice field ecosystem is constitutively determined by the monoculture of rice plants and concomitant water saturated soil. Short periods of drainage occur only during rice harvest and field preparation. In this thesis two soils from different locations (Vercelli, Italy (**Chapter 2**); Los Banos, Philippines (**Chapter 3**)) cultured with two special rice plant varieties (*Oryza sativa*: Koral (**Chapter 2**); NSIC Rc222 (**Chapter 3**)) revealed comparable patterns during rice plant growth. However, minor changes in the community were observed concentrating on specific microbial lineages at explicit locations influenced by the rice plant. These results strengthen the assumption of a highly adapted overall microbial community in rice field soil.

5.2 Crop rotation

Crop rotation between flooded rice and upland plants are gaining more and more importance in the world's major-rice producing region Asia. Since the world's population is anticipated to increase, the need for cultivating staple foods like rice will further enlarge (Van Nguyen and Ferrero, 2006). However, rice cultivation is accompanied with intensive water consumption (Tuong *et al*., 2005) and so farmers are anticipated to face periods of water scarcity (Bouman *et al*., 2005). The upland crop maize is commonly cultivated in the Philippine uplands (Bertomeu, 2012) and its demand is increasing due to poultry and biofuel production (Weller *et al*., 2015a). Further, maize is a cash crop and therefore maize farming is economical which leads to notably realized rice–maize rotation systems (Timsina *et al*., 2010). The impact of crop rotations on crop yields and health were studied extensively in the past revealing positive effects on both (Xuan *et al*., 2012; Peters *et al*., 2003; Mendes *et al*., 2011).

However, the impacts on the rice field soil inhabiting microbial communities and their activity are rarely studied and rather focused on either the archaeal or the bacterial community (Table 5.1). In this thesis a rice-maize crop rotation was investigated focusing on both, the resident and active archaeal as well as bacterial community. Therefore, a crop rotational field rotating irrigated rice and upland maize was studied in comparison to a field only cultivated with rice. The impact of rotation was thereby investigated in detail during the first season of rotation revealing immediately occurring effects (**Chapter 3**) and over two years of rotation screening for long term effects (**Chapter 4**).

Studies focusing on crop rotations including flooded rice can be divided in two categories: (I) native upland systems in which flooded rice is implemented and (II) flooded rice field systems rotated with plants under upland conditions. These categories have to be differentiated based on their field management history, meaning whether these fields were historically under drained or flooded conditions. For crop rotation systems out of category (I) it was shown that the bacterial community changed in composition, especially anaerobes were found in higher relative abundance with rice cultivation and accompanied water saturated conditions enabling anoxic niches (Table 5.1; Lopes *et al*., 2014; Fernandez Scavino *et al*., 2013; Zhao *et al*., 2014b). In a pasture-rice rotation it was shown that once

pasture soil has been rotated with flooded rice a constant methanogenic microbial community was established, even if the flooded conditions were followed by 4 years of upland conditions (Fernandez Scavino *et al*., 2013). These studies indicate that the introduction of irrigated crops like rice in drained upland ecosystems leads to the enrichment of anaerobic archaea and bacteria, which tend to withstand periods of drainage once they established.

Since communities of anaerobic archaea and bacteria seem to establish relatively fast and display the ability to resist periods of drainage, the question can be raised how innate anaerobic communities are impacted by the introduction of upland plants and the accompanied drainage (category II). Several studies focused on crop rotation systems of flooded rice and upland crop in Asian countries such as Japan, China, Vietnam and Philippines (Table 5.1; Asakawa and Hayano, 1995; Liu *et al*., 2015; Zhao *et al*., 2014 a; Watanabe *et al*., 2006, 2009; Xuan *et al*., 2012; **Chapter 3,4**). Different crop rotational systems were examined such as a multiannual rice-soybean rotation, where the fields were cultivated alternating with irrigated rice for several years followed by the cultivation of soybean for several years (Liu *et al*., 2015). Thereby, the microbial community is exposed to extended periods (years) of drained conditions in multiannual rotation systems. Liu *et al*. (2015) showed that the rotation affected the methanogenic community negatively indicated by a decrease of the overall methanogenic abundance and the relative abundance of some species out of the *Methanosarcinales*.

In rotation systems which alternate crops in two or three seasons per year (bi-, triannual systems) the time of drainage and possibly increased oxygen exposure is rather limited (several months). The effects of rotations of flooded rice with wheat, maize or mungbean resulted in differences in the bacterial community (Table 5.1; Xuan *et al*., 2012; Zhao *et al*., 2014a). These studies showed dynamics in the bacterial community composition in the rotational fields possibly due to their ecophysiology. However, these studies only monitored the effect of the crop rotation in already established rotation systems which were managed for several years (Xuan *et al*., 2012: 7 years; Zhao *et al*., 2014a: 2 years). In this thesis the effect of the first introduction of the upland plant maize on the microbial community inhabiting the rice field soil was investigated (**Chapter 3**). To our knowledge

Chapter 3 was the first study investigating the effect of the primary introduction of maize into the rice field soil. Thereby we showed that the bacterial community was only slightly affected and seemed to respond stronger to drainage than to the introduction of the maize plant. Long term effects on the bacterial community were also not very dramatic (**Chapter 4**). Only *Acidobacteria* and *Anaeromyxobacter* spp. were enriched in the rotational fields possibly due to the increased oxygen inflow, while members of anaerobic *Chloroflexi* and sulfite reducing members of *Deltaproteobacteria* were found in higher abundance in the continuous rice fields indicating a negative effect of drainage on these anaerobic bacteria in the rotational fields. This raises the question why the introduction of maize caused only minor changes in the Philippine rice field soil while different rice-upland rotations revealed even stronger changes in the bacterial community (Table 5.1; Xuan *et al*., 2012; Zhao *et al*., 2014a). These differences are not easily explained, however it is noteworthy that the discussed rotations differed in substantial aspects from each other such as rotation history, soil type/texture and plant species.

The impact of crop rotations on the methanogenic community has been studied mainly in Japanese rice fields (Table 5.1; Asakawa and Hayano, 1995; Watanabe *et al*., 2006, 2009). In these rice-wheat rotations the resident community was unaffected in abundance and the composition was mainly stable, as only some methanogens differed between rotation and control fields (Table 5.1; Watanabe *et al*., 2006, 2009). In this thesis the immediate effect of maize cultivation on the archaeal community was likewise minor since only *Methanosarcinaceae* and Soil Crenarchaeotic Group were impacted (**Chapter 3**). In the season following the first maize cultivation dramatic changes in the archaeal community were observed (**Chapter 4**). It may be possible that the changes occurred in the time between our samplings. Between the sampling time points the microbes were exposed to upland conditions for nearly three months. Thereby potential aerobic Archaea such as *Crenarchaeota*/*Thaumarchaeota* increased their relative abundance while anaerobic methanogens decreased. This seems to be very dramatic in comparison with the observations from the Japanese rice fields (Asakawa and Hayano, 1995; Watanabe *et al*., 2006, 2009). However, in the investigated fields in these studies were subjected to crop rotation since 1963 (Watanabe *et al*., 2006, 2009) and Asakawa and Hayano (1995) started sampling in the second phase of upland conditions. In contrast, the Philippine study site investigated in this

thesis was cultivated with flooded rice for over 20 years before the first introduction of maize (Weller *et al*., 2015a) which was monitored in **Chapter 3**. Therefore it may be that the microbial community has first to adapt to the stress of crop rotation (**Chapter 4**) before the archaeal/methanogenic community reveals the relative stability observed in previous studies monitoring rice-wheat and pasture-rice crop rotations (Table 5.1; Asakawa and Hayano, 1995; Fernandez Scavino *et al*., 2013; Watanabe *et al*., 2006, 2009). Indeed this may be indicated by Liu *et al*. (2015) monitoring rice-soybean crop rotation in comparison to only rice fields at two different locations in Japan. Thereby, the abundance and the composition of methanogens differed between the rotational fields and the control rice fields, but within the rotational fields the numbers and of methanogens and their community composition was unaltered regardless of managing the fields as flooded rice field or as upland soybean fields (Liu *et al*., 2015). However, further extended studies are needed to confirm this hypothesis.

All together it was shown that the bacterial and archaeal communities respond in different ways to crop rotations and changes in field management. In this thesis a detailed analysis of the impact of the introduction of upland maize into rice dominated landscape was given, revealing minor immediate effects (**Chapter 3**) and stronger pronounced long term effects (**Chapter 4**) on the microbial community. However, extended observation of the impact of crop rotation on the microbial communities is needed to understand the ability of anaerobic archaeal and bacterial communities to survive these extended periods of drainage and oxygen exposure.

Table 5.1 Summary of studies investigating the impact of rice crop rotational systems on the archaeal and bacterial community.

Rotation systems		***Archaea***		***Bacteria***		***Reference***
Pattern	***Crops***	***Abundance***	***Composition***	***Abundance***	***Composition***	
Upland-irrigated (category I)						
multiannual	Alfalfa-rice	-	-	-	Increase of anaerobes with rice Second rice cultivation resulted in stronger change	Lopes *et al*., 2014
multiannual	Pasture-rice	Higher methanogenic archaea in rotational fields	Minor changes in rotational fields	-	Different community patterns in rotational and pasture fields	Fernandez Scavino *et al*., 2013
biannual	Wheat-rice	-	-	-	Minor effect of crop rotation Increase of anaerobes and diversity with rice cultivation	Zhao *et al*., 2014b
Irrigated-upland (category II)						
multiannual	Rice-soybean	Methanogens decreases in rotational fields	Composition changes: Some *Methanosarcinales* negatively affected	-	-	Liu *et al*., 2015
biannual	Rice-wheat	Constant numbers of methanogens in rotation	-	-	-	Asakawa and Hayano, 1995
biannual	Rice-wheat	-	-	-	Shift in community Increase of anaerobes with rice	Zhao *et al*., 2014a
biannual	Rice-wheat	-	Composition was unaffected by rotation	-	-	Watanabe *et al*., 2006
biannual	Rice-wheat	Similar numbers of resident and higher active during rice cultivation	Methanogenic community differed on species level	-	-	Watanabe *et al*., 2009
biannual	Rice-maize	Decrease of resident archaea with maize cultivation	More *Thaumarchaeota* less *Euryarchaeota* in rotational fields	Decrease of resident bacteria with maize cultivation	Minor changes in bacterial community composition	**Chapter 3, 4**
triannual	Rice-maize-rice	-	-	-	Diversity and composition differed from rice fields	Xuan *et al*., 2012
triannual	Rice-mungbean-rice	-	-	-	Diversity and composition differed from rice fields	Xuan *et al*., 2012

Biannual: two seasons per year, mainly winter (upland crop) and sommer (irrigated crop); triannual: three seasons per year; multiannual: only one crop per year, then change with rotational crop; -: not investigated

5.3 Increased rRNA levels - a potential stress response?

In this thesis resident and active communities of bacteria and archaea were studied by monitoring the abundance (qPCR) and composition (454 pyrosequencing) targeting the 16S rDNA respectively 16S rRNA. Here, the active community members were defined by their ribosomal RNA, since the number of ribosomes is considered to reflect activity (e.g. Egert *et al*., 2011.). However, it remains unknown whether the microbes with increased numbers of ribosomes really express a higher level of activity. Recently, the detection of rRNA and its implications were critically illuminated (Blazewicz *et al*., 2013). Here, during drainage of Philippine rice field soil an increased RNA/DNA ratios were observed for both, bacteria and archaea, in comparison to a flooded rice field (**Chapter 3**). The abundance of the 16S rDNA decreased whereas the abundance of ribosomal RNA was unaltered indicating the maintenance of a high level of ribosomal RNA. This phenomenon was interpreted as stress response to the drainage and as preparedness for activity when conditions improve. Supporting similar increased ratios of rRNA/rDNA have also been observed in non-flooded Japanese rice fields (Watanabe *et al*., 2007). Contrary, decreasing rRNA levels with drainage, were reported for a Japanese rice field (Itoh *et al*., 2013). It has been already discussed that the increased RNA/DNA ratio can be interpreted as an enhanced activity since the microbes possibly were protected in anaerobic microniches along with easily available nutrients and/or increased temperature (**Chapter 3**).

In the following dry season increased RNA/DNA ratios were observed for members of the archaeal community (**Chapter 4**). Again, these increased ratios were found under unfavourable conditions i.e. for the strict anaerobic methanogens under drainage during maize cultivation. This finding supports the suggestion that the increase of ribosomal RNA may be a stress reaction. Indeed it has been shown that dormant cells can contain more rRNA than vegetative cells (Sukenik *et al*., 2012). Non-dormant pure cultures retained ribosome levels greater than current synthesis requirements (Koch 1971, Alton and Koch, 1974; Flärdh *et al*., 1992) indicating better adaption to changing conditions by enabling the shift of metabolic functions if needed (Blazewicz *et al*., 2013). Recently, the maintenance of 16 rRNA levels was observed for methanotroph pure cultures under extended periods of starvation (Brandt *et al*., in preparation). Further, ammonia-oxidizers showed unaltered

ribosome abundance with starvation (Morgenroth *et al*., 2000) or inhibition (Schmid *et al*., 2000; Wagner *et al*., 1995).

Supporting our hypothesis that retained or even increased ribosome abundance under starvation/stress serves as preparedness for improving conditions it was described that mRNAs are maintained in the cell by revealing extended half life times. The global mRNA half-life in bacterial and archaeal pure cultures ranges between 5-10 minutes (Hambraeus *et al*., 2003; Selinger *et al*., 2003; Andersson *et al*., 2006), however some mRNAs have half-lives of more than 15 minutes (Hambraeus *et al*., 2003; Brandt *et al*., in preparation). In *Lactococcus lactis* the stability of most mRNAs increased with decreasing growth rate (Dressaire *et al*., 2013). Investigating transcripts of functional marker genes coding for enzymes important for the metabolism under starvation or stress conditions revealed interesting patterns. For instance, a basal pool of *pmoA* mRNA, encoding a subunit of the methane monooxygenase, was maintained in methanotrophs under starvation (Brandt *et al*., in preparation). Further, incubating Philippine rice field soil under drained conditions also resulted in a decrease of *mcrA* mRNA level during the first 48 h, which was then stabilized for more than 60 days (Breidenbach *et al*., in preparation; data not shown). Together, the ability to maintain basal levels of mRNA under unfavourable conditions in turn indicates the need for maintaining high amounts of ribosomes as sites of biological protein synthesis.

In conclusion, the maintenance of high numbers of ribosomes under drained conditions may function as stress response and potential preparedness for improving conditions as it enables spontaneous shifts in metabolic functions by providing immediate protein synthesis potential.

5.4 Outlook

In this thesis we provided evidence that the rice plants are able to influence the microbial community in the soil surrounding the roots. In a greenhouse experiment we were able to identify bacterial lineages which were stimulated in the presence of the rice plant (**Chapter 2**). Further research is needed to directly identify the microbes which assimilate the carbon compounds provided by the plant. Several stable isotope tracer studies were already successful indentifying bacterial and archaeal lineages assimilating these carbon compounds (Lu and Conrad, 2006; Zhu *et al*., 2014; Hernández *et al*., 2015). However, these studies only investigated one particular time point during plant growth. Therefore stable isotope tracers experiments following the growth stages are still missing. Further, dedicated analysis of the active microbes in the rhizosphere can be conducted. Methods such as metatranscriptomics and metaproteomics allow the detection of the active microbes by tracking their total RNA and the proteins. Another aspect to follow may be the analysis of the compounds excreted by the rice plants. If these were identified, experiments using stable isotope or radioactive labeled replicates of these compounds could be conducted. Incubation of labeled compounds with rice field soil enables the identification of the microorganisms involved in their degradation. Lastly, the influence of several microbial lineages on the plant can be investigated to identify plant growth promoting lineages. Therefore, more sophisticated experiments with plants growing in soil mimicking material under addition of several defined microbial communities are needed. These experiments also have the potential to indentify further microbe-plant interactions.

The second part of this thesis concentrated on the impact of changes in field management such as crop rotation on the microbial community in rice field soil. We showed that the introduction of maize plants cultivated as upland crop into a flooded rice ecosystem resulted in minor short term (**Chapter 3**) and strong long term effects (**Chapter 4**). The main effect of the crop rotation was found in the archaeal community as it shifted from mainly anaerobic methanogens to aerobic *Thaumarchaeota*. Contrary, many other studies showed that the archaeal community is relatively stable during crop rotations. To our best knowledge this thesis is the first investigation of a crop rotation from the primary introduction of the unfamiliar plant and upland cultivation to the soil. Equally to previous

studies we anticipated that the community in the Philippine soil will also reveal such stability after adaption to the stress of crop rotation, which may last for several seasons. To prove this, long term experiments (several years) monitoring the crop rotational at the field site in the Philippines are needed. Within the multidisciplinary research project ”Introduction of non-flooded crops in rice-dominated landscapes and its impact on carbon, nitrogen and water cycles (ICON)” we will be able to connect the gained knowledge in soil microbiology with measured nutrient fluxes at the field site. It is of high importance to connect processes with microbial activity such as transcription or protein assembling. New techniques such as single-cell Raman spectroscopy in combination with stable isotope probing offer culture-independent investigations of genetic functions and physiology of unculturable microorganisms in an ecosystem (Li *et al*., 2014). In this thesis we reported relatively high numbers of archaeal lineages taxonomically assigned as GOM Arc I. We speculated that these were actually ANME-2d species capable of anaerobic methane oxidation. One possibly approach to test this hypothesis would be to spot these archaea with specific fluorescence *in situ* hybridization (FISH) probes and to investigate their genetic functions and physiology in the soil using Raman. However, more classical approaches such as enrichment under defined conditions aiming for pure cultures of these species may also serve this purpose. Progress in the identification of anaerobic methane oxidizers may further help identifying the importance of this mechanism on field scale. The techniques mentioned above may also serve to prove our hypothesis that the increased number of ribosomes per cell serves as stress response. Therefore an experiment incubating Philippine rice field soil under drainage and rewetting stress might be conducted.

On a more global scale it is important to follow the practices used by farmers in the rice fields to reduce water consumption and mitigate CH_4 emissions. Understanding the mechanisms underlying the effects resulting from these practices is highly significant. Besides the crop rotation systems a catalog of water reducing techniques is implemented already in the daily routine such as intermittent drainage, mid-season drainage and alternate wetting and drying. Besides the changes in water levels soil amendments (e.g. phosphogypsum, silicate fertilizer) can further mitigate greenhouse gas emissions and improve rice yields (Richards and Sander, 2014). These techniques are suggested in

implementation guidance for policymakers and investors. However, the underlying processes are not fully understood and further research is needed.

5.5 References

Alton, T. H., and Koch, A. L. (1974). Unused protein synthetic capacity of *Escherichia coli* grown in phosphate-limited chemostats. *J. Mol. Biol*. 86, 1-9.

Andersson, A. F., Lundgren, M., Eriksson, S., Rosenlund, M., Bernander, R., and Nilsson, P. (2006). Global analysis of mRNA stability in the archaeon *Sulfolobus*. *Genome Biol.* 7, R99. doi:10.1186/gb-2006-7-10-r99.

Asakawa, S. and Hayano, K. (1995). Population of methanogenic bacteria in paddy field soil under double cropping conditions (rice-wheat). *Biol. Fertil. Soils* 20, 113-117.

Aulakh, M. S., Wassmann, R., Bueno, C., Kreuzwieser, J., and Rennenberg, H. (2001). Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biol*. 3, 139-148. doi: 10.1055/s-2001-12905.

Bertomeu, M. (2012). Growth and yield of maize and timber trees in smallholder agroforestry systems in Claveria, northern Mindanao, Philippines. *Agrofor. Syst.* 84, 73-87.

Blazewicz, S. J., Barnard, R. L., Daly, R. A., and Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. *ISME J*. 7, 2061-2068.

Bouman, B. A. M., Peng, S., Castaneda, A. R., and Visperas, R. M. (2005). Yield and water use of irrigated tropical aerobic rice systems. *Agric. Water Manag*, 74, 87-105.

Brandt, F.B., Pommerenke B., and Dumont, M.G. Continued presence of *pmoA* mRNA and 16S rRNA in aerobic methanotrophs under anoxic conditions without methane. *In preparation*

Breidenbach, B., Blaser, M., Freude, C., Klose, M., and Conrad, R. Abundance of methanogenic marker (*mcrA*) transcripts in rice-maize crop rotation and long term drainage. *In preparation*

Dressaire, C., Picard, F., Redon, E., Loubière, P., Queinnec, I., Girbal, L., and Cocaign-Bousquet, M. (2013). Role of mRNA stability during bacterial adaptation. *PloS One* 8, e59059. doi:10.1371/journal.pone.0059059.

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., *et al*. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci.* 112, E911-E920.

Egert, M., Schmidt, I., Höhne, H. M., Lachnit, T., Schmitz, R. A., and Breves, R. (2011). rRNA-based profiling of bacteria in the axilla of healthy males suggests right–left asymmetry in bacterial activity. *FEMS Microbiol. Ecol.* 77, 146-153.

Fernandez Scavino, A., Ji, Y., Pump, J., Klose, M., Claus, P., and Conrad, R. (2013). Structure and function of the methanogenic microbial communities in Uruguayan soils shifted between pasture and irrigated rice fields. *Environ. Microbiol*. 15, 2588-2602.

Flärdh, K., Cohen, P. S., and Kjelleberg, S. (1992). Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. *J. Bacteriol.* 174, 6780-6788.

Hambraeus, G., Wachenfeldt, C., and Hederstedt, L. (2003). Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol. Gen. Genomics* 269, 706-714.

Hernández, M., Dumont, M. G., Yuan, Q., and Conrad, R. (2015). Different bacterial populations associated with the roots and rhizosphere of rice incorporate plant-derived carbon. *Appl. Environ. Microbiol.* doi:10.1128/AEM.03209-14

Itoh, H., Ishii, S., Shiratori, Y., Oshima, K., Otsuka, S., Hattori, M., and Senoo, K. (2013). Seasonal transition of active bacterial and archaeal communities in relation to water management in paddy soils. *Microbes Environ*. 28, 370-380.

Kimura, M., Murase, J., and Lu, Y. H. (2004). Carbon cycling in rice field ecosystems in the context of input, decomposition and trans-location of organic materials and the fates of their end prod-ucts (CO_2 and CH_4). *Soil Biol. Biochem.* 36, 1399-1416.

Koch, A. L. (1971). The adaptive responses of *Escherichia coli* to a feast and famine existence. *Adv. Microb. Physiol*. 6, 147-217.

Li, M., Boardman, D. G., Ward, A., and Huang, W. E. (2014). "Single-cell Raman sorting", in: *Environmental Microbiology*, Humana Press, Springer New York, USA, 147-153.

Liu, D., Ishikawa, H., Nishida, M., Tsuchiya, K., Takahashi, T., Kimura, M., and Asakawa, S. (2015). Effect of paddy-upland rotation on methanogenic archaeal community structure in paddy field soil. *Microb. Ecol*. 69, 160-168.

Lopes, A. R., Manaia, C. M., and Nunes, O. C. (2014). Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. *FEMS Microbiol. Ecol.* 87, 650-663.

Lu, Y., and Conrad, R. (2005). In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* 309, 1088-1090.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., *et al*. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, 332, 1097-1100.

Morgenroth, E., Obermayer, A., Arnold, E., Brühl, A., Wagner, M., and Wilderer, P. A. (2000). Effect of long term idle periods on the performance of sequencing batch reactors. *Wat. Sci. Technol.* 41, 105-113.

Peters, R. D., Sturz, A. V., Carter, M. R., and Sanderson, J. B. (2003). Developing disease-suppressive soils through crop rotation and tillage management practices. *Soil Til. Res.* 72, 181-192.

Richards, M., and Sander, B. O. (2014). "Alternate wetting and drying in irrigated rice", in: *CSA Practice Brief*, CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS), Copenhagen, Denmark, www.ccafs.cgiar.org.

Schmid, M., Twachtmann, U., Klein, M., Strous, M., Juretschko, S., Jetten, M., *et al*. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Syst. Appl. Microbiol*. 23, 93-106.

Selinger, D. W., Saxena, R. M., Cheung, K. J., Church, G. M., and Rosenow, C. (2003). Global RNA half-life analysis in *Escherichia coli* reveals positional patterns of transcript degradation. *Genome Res*. 13, 216-223.

Sukenik, A., Kaplan-Levy, R. N., Welch, J. M., and Post, A. F. (2012). Massive multiplication of genome and ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalis-porum* (*Cyanobacteria*). *ISME J*. 6, 670–679. doi: 10.1038/ismej.2011.128.

Timsina, J., Jat, M. L., and Majumdar, K. (2010). Rice-maize systems of South Asia: current status, future prospects and research priorities for nutrient management. *Plant Soil* 335, 65-82.

Tuong, T. P., Bouman, B. A. M., and Mortimer, M. (2005). More rice, less water-integrated approaches for increasing water productivity in irrigated rice-based systems in Asia. *Plant Prod. Sci.* 8, 231-241.

Van Nguyen, N., and Ferrero, A. (2006). Meeting the challenges of global rice production. *Paddy Water Environ*. 4, 1-9. doi: 10.1007/s10333-005-0031-5.

Wagner, M., Rath, G., Amann, R., Koops, H. P., and Schleifer K. H. (1995). *In situ* identification of ammonia oxidizing bacteria. *Syst. Appl. Microbiol.* 18, 251–264.

Watanabe, T., Kimura, M., and Asakawa, S. (2006) Community structure of methanogenic archaea in paddy field soil under double cropping (rice-wheat). *Soil Biol. Biochem.* 38, 1264-1274.

Watanabe, T., Kimura, M., and Asakawa, S. (2007). Dynamics of methanogenic archaeal communities based on rRNA analysis and their relation to methanogenic activity in Japanese paddy field soils. *Soil Biol. Biochem.* 39, 2877-2887.

Watanabe, T., Kimura, M. and Asakawa, S. (2009). Distinct members of a stable methanogenic archaeal community transcribe *mcrA* genes under flooded and drained conditions in Japanese paddy field soil. *Soil Biol. Biochem*. 41, 276-285.

Xuan, D.T., Guong, V. T., Rosling, A., Alström, S., Chai, B., and Högberg, N. (2012). Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biol. Fertil. Soils* 48, 217-225.

Zhao, J., Ni, T., Li, Y., Xiong, W., Ran, W., Shen, B., *et al*. (2014a). Responses of bacterial communities in Arable soils in a rice-wheat cropping system to different fertilizer regimes and sampling times. *PloS One* 9, e85301. doi:10.1371/journal.pone.0085301.

Zhao, J., Zhang, R., Xue, C., Xun, W., Sun, L., Xu, Y., and Shen, Q. (2014b). Pyrosequencing reveals contrasting soil bacterial diversity and community structure of two main winter wheat cropping systems in China. *Microb. Ecol*. 67, 443-453.

Zhu, W., Lu, H., Hill, J., Guo, X., Wang, H., and Wu, W. (2014). ^{13}C pulse-chase labeling comparative assessment of the active methanogenic archaeal community composition in the transgenic and nontransgenic parental rice rhizospheres *FEMS Microbiol. Ecol.* 87, 746-756.

Appendices

Wissenschaftliche Publikationen

Breidenbach B, Brandt FB, Brenzinger K, and Conrad R (2014) Impact of short-term storage temperature on determination of microbial community composition and abundance in aerated forest soil and anoxic pond sediment samples. *Systematic and applied microbiology*, 37(8), 570-577.

Breidenbach B and Conrad R (2015) Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and effect of drainage. *Frontiers in Microbiology* 5:752. doi: 10.3389/fmicb.2014.00752

Beiträge zu wissenschaftlichen Tagungen

Annual Conference of the Association for General and Applied Microbiology (VAAM), 2009

Breidenbach B, Cabezas A and Friedrich MW *Effect of sediment microbial fuel cells on methane emission from rice paddies* (Poster Presentation)

Annual Conference of the Association for General and Applied Microbiology (VAAM), 2011

Breidenbach B, Pump J and Dumont MG *Investigation into the effect of growth stages on the rhizospheric microbial community of rice plants* (Poster Presentation)

5TH Congress of European Microbiologists (FEMS), 2013

Breidenbach B, Blaser M and Conrad R *Effect of Crop Rotation on Active Microbial Community Involved in Methane Production* (Poster Presentation)

Marburg Meeting on Microbiology, 2014

Breidenbach B, Blaser M and Conrad R *Impact of Crop Rotation on Active Microbial Community Involved in Methane Formation* (Poster Presentation)

International Workshop 'Biogeochemistry of submerged agro-ecosystems: Properties, processes, cycles and functions', 2014

Breidenbach B, Blaser M, Pledl P, Schneider B and Conrad R *Effect of Crop Rotation on the Active Methanogens in Rice Fields* (Poster Presentation)

International Workshop ‘Biogeochemistry of submerged agro-ecosystems: Properties, processes, cycles and functions’, 2014

Blaser M, Hahn A, Freude C, *Breidenbach B* and Conrad R *Deciphering methanogenic pathways in Philippine rice field soil using natural abundance of stable carbon isotopes* (Poster Presentation)

International Workshop ‘Biogeochemistry of submerged agro-ecosystems: Properties, processes, cycles and functions’, 2014

Breidenbach B and Conrad R *Active microbial communities under crop rotation in a rice dominated landscape* (Oral Presentation)

Annual Conference of the Association for General and Applied Microbiology (VAAM), 2015

Breidenbach B and Conrad R *Seasonal dynamics of bacterial and archaeal methanogenic communities in flooded rice fields and the effect of drainage and crop rotation* (Poster Presentation)

Abgrenzung der Eigenleistung

Diese Arbeit wurde im Rahmen des Forschungsprojektes ICON (‘Introducing Non-Flooded Crops in Rice-Dominated Landscapes: Impacts on Carbon, Nitrogen and Water Cycles [ICON]’) angefertigt, welches von der Deutschen Forschungsgemeinschaft (DFG) finanziert wurde. Das Haupthema dieser Arbeit wurde von meinem Betreuer, Prof. Dr. Ralf Conrad, konzipiert. Soweit nicht anders erwähnt, wurden alle Experimente von mir selbst geplant und durchgeführt, sowie anschließend in Form eines Manuskriptes ausgewertet. Das Verfassen des Manuskriptes erfolgte in Zusammenarbeit mit meinem Betreuer, Prof. Dr. Ralf Conrad.

Erklärung

Ich versichere, dass ich meine Dissertation

‘Rice plants, drainage and crop rotation influence the methanogenic community in rice field soil’

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg März 2015

Danksagung

An erster Stelle möchte ich mich herzlich bei meinem Doktorvater Herrn Prof. Dr. Ralf Conrad bedanken, der mir die Gelegenheit gegeben hat, diese Doktorarbeit unter seiner Anleitung anzufertigen. Ich bedanke mich für alle Ratschläge und Diskussionen und für die Freiheit mit der ich dieses Projekt bearbeiten durfte.

Des Weiteren bedanke ich mich herzlich bei Herrn Prof. Dr. Michael Bölker für die Übernahme des Zweitgutachtens.

Großer Dank gebührt Herrn Prof. Dr. Erhard Bremer und Herrn Prof. Dr. Diethart Matthies für ihre Beteiligung an meiner Prüfungskommission sowie meinem Thesis-Komitee, bestehend aus Herr Prof. Dr. Peter Frenzel, Herr Prof. Dr. Michael Bölker und Herr Prof. Dr. Erhard Bremer für hilfreiche Diskussionen und Ratschläge.

Ein besonderer Dank gilt Dr. Martin Blaser für anregende Diskussionen (auch abseits der Forschung), fortwährende Unterstützung und organisatorische Arbeiten im ICON-Projekt.

Ich bedanke mich herzlich bei Dr. Marc Dumont für seine Unterstützung, kritische Diskussionen, Motivation und das vorgelebte Durchhaltevermögen.

Ein besonderes Dankschön an alle, die mich mit großer Sorgfalt bei dieser Arbeit tatkräftig unterstüzt haben: Melanie Klose, Christoph Freude, Belinda Schneider, Peter Claus und Alexandra Hahn. Allen Mitgliedern der AG Conrad gilt ein besonderer Dank für die durchweg positive Stimmung und die nette Arbeitsatmosphäre. Ebenso bedanke ich mich bei allen Mitgliedern der Abteilung Biogeochemie für eine gute Arbeitsatmosphäre.

Ein großer Dank an alle, die diese Arbeit korrekturgelesen haben, allen voran Franziska Brandt, Dr. Martin Blaser, Dr. Judith Pump und Dr. Karen Rossmassler.

Ein besonderer nahezu wahlloser Dank gilt Kristof Brenzinger und Franziska Brandt. Danke für die unendlichen Stunden Spaß innerhalb und außerhalb des Labors. Ohne euch wäre diese Arbeit nicht entstanden. DÜF.

Ein weiterer Dank gilt den Menschen, die meine Zeit außerhalb des Labors in Marburg zu einem wahren Vergnügen gemacht haben: Daniel, Simon, Tillmann, Joss, Max, Basti, Franziska, Judith, Kristof und Suse mit Noah, Marcela und Marc mit Emma und Mathilda.

Besonders möchte ich mich bei meinen Eltern bedanken, ohne ihre Untestützung und Liebe wäre diese Arbeit nicht möglich gewesen.

Zu guter Letzt möchte ich mich bei Franziska bedanken: Tack för din positivt sätt, ditt skratt, ditt stöd och din kärlek.