Colonization of the rice rhizosphere by microbial communities involved in the syntrophic degradation of rhizodeposits to methane

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“So much universe, and so little time.“

- Terry Pratchett

Table of contents

List of abbreviations

ANOVA	analysis of variance
bp	base pairs
BSA	bovine serum albumin
CCA	canonical correspondence analysis
DCA	detrended correspondence analysis
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
FID	flame ionization detector
GC	gas chromatography
HPLC	high performance liquid chromatography
IRMS	isotope ratio mass spectrometer
IRRI	International Rice Research Institute
KOSI	Kompetenzzentrum Stabile Isotope
mcr	methyl-coenzyme M reductase
mcrA	small α subunit of the methyl-coenzyme M reductase
misc.	miscellaneous
MMO	methane monooxygenase
MOB	methane-oxidizing bacteria
NCBI	national center for biotechnology information
nt	nucleotide
OTU	operational taxonomic unit
PCR	polymerase chain reaction
pMMO	particular methane monooxygenase

pmoA	subunit of the particular methane monooxygenase
ppm_v	parts per million volume
qPCR	real-time quantitative polymerase chain reaction
RI	refractive index
RNA	ribonucleic acid
ROL	radial oxygen loss
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SCFA	short chain fatty acids
SD	standard deviation
SIP	stable isotope probing
sp.	species
t-RFLP	terminal restriction fragment length polymorphism analysis
UV	ultraviolet
vs.	versus

Summary

Roots represent the primary site of direct interaction between rice plants and soil microorganisms. The influence of the plant on the soil microbial community includes the translocation of photosynthetically fixed carbon into the rhizosphere as rhizodeposition. This extends to the rhizosphere of rice plants, which is colonized by a syntrophic microbial community, which in turn is able to degrade root derived carbon to methane. Each plant species is thought to select a specific microbial community composition as root microbiome. A general understanding of microbial colonization of the rice rhizosphere and the consequential impact on the emission of methane originating from rhizodeposits is still uncertain, since the majority of the studies have so far focused exclusively on rice roots planted in rice paddy soils.

Therefore, we used different initial soil microbial communities available for colonization of the rice roots. In order to do this, an inert sand-vermiculite matrix was inoculated with rice paddy soil and digested sludge, respectively, and was afterwards planted with rice. The microbial activity essential for the formation of methane from those soil-systems was tested in pre-experiments and the colonization of the rice rhizosphere was determined afterwards in plant-soil microcosms. Each of the microcosms possessed an individually structured microbial community, which served as a seed bank for the colonization of the rice roots. We analyzed the impact of the community composition on the emission of methane by combining $^{13}CO_2$ pulse-labeling with illumina sequencing and quantitative PCR, targeting the 16S rRNA as phylogenetic-, as well as *mcrA* and *pmoA* as functional marker genes for methanogenic archaea and methane-oxidizing bacteria.

The degradation of rhizodeposits to methane in the different microcosms was dependent on the bacterial and methanogenic community structure, but not on their absolute abundance in the rhizosphere. Like the colonization of the rhizosphere by bacteria and methanogenic archaea, the translocation of photosynthetically fixed carbon depended upon the initial microbial communities. Nevertheless, the rice rhizosphere was found to be a distinct habitat for bacteria and methanogenic archaea.

We were able to identify a methanogenic community which was linked to the degradation of rhizodeposits to methane across the rhizosphere of all microcosms. Besides hydrogenotrophic *Methanocella* and *Methanobacteriaceae*, acetoclastic *Methanosaeta* could also be assigned to this community. Nevertheless, most methanogens which contribute to the emission of methane originating from root derived carbon were found to belong to those with a hydrogenotrophic pathway. Within the methanogenic community linked to the formation of methane from rhizodeposits, the root surface was mainly colonized by hydrogenotrophic methanogens, while those able to perform acetoclastic methanogenesis were highly abundant in the rhizospheric soil. This was true of the colonization of the rice rhizosphere by methanogenic archaea in general.

Furthermore, we were able to identify methanogens which were ubiquitous in the rhizosphere of all microcosms. Those were considered as methanogenic community selected by the rice plant on its roots. Representatives of *Methanobacteriaceae*, *Methanosaeta* and *Methanosarcina* colonized the overall rhizosphere, while *Methanocella* were found to be present in the rhizospheric soil of all microcosms. In addition to this, all methanogenic archaea which were linked to the degradation of root derived carbon to methane also belonged to this root associated community. Hence, the methanogenic community selected on the rice root also contributed to the formation of methane from rhizodeposits.

Besides methanogens, we were also able to identify certain bacterial groups, which are linked to the degradation of root derived carbon to methane. These includes representatives of *Kineosporiaceae*, *Anaeromyxobacter*, *Bradyrhizobium*, and *Bacteroidales*. A higher abundance of *Kineosporiaceae* also resulted in an increased conversion of root derived carbon compounds to acetate, CO_2, and propionate. Therefore, at least the family of *Kineosporiaceae* was thought to be actively involved in the degradation of rhizodeposition to precursors for methanogenesis. Nevertheless, we were not able to determine bacteria which contributed to the emission of methane originating from root derived carbon and which were ubiquitous in the rhizosphere of all soil-systems. This resulted from the fact that the microbial community structure of the rhizosphere also depended on the initial pool of microorganisms available for root colonization.

Zusammenfassung

Die Pflanzenwurzel stellt die primäre Zone für Interaktionen zwischen Reispflanzen und Bodenmikroorganismen dar. Pflanzen nehmen unter anderem durch Translokation von photosynthetisch fixiertem Kohlenstoff in die Rhizosphäre als Rhizodeposition Einfluss auf die im Boden vorkommenden mikrobiellen Gemeinschaften. Dies gilt auch für Reiswurzeln, welche von einer syntrophen mikrobiellen Lebensgemeinschaft besiedelt sind, die Methan aus wurzelbürtigen organischen Kohlenstoffverbindungen bilden kann. Es konnte bereits gezeigt werden, dass jede Pflanzenart eine spezifische mikrobielle Gemeinschaft an ihren Wurzeln selektiert. Ein generelles Verständnis über die Kolonisierung der Rhizosphäre von Reispflanzen und der sich daraus ergebende Einfluss auf den Abbau von Rhizodepositen zu Methan fehlt jedoch, da der Großteil der veröffentlichen Studien das Augenmerk ausschließlich auf solche Wurzeln legt, die auch in Reisfeldböden herangewachsen sind.

Daher haben wir für diese Studie verschiedene mikrobielle Ausgangs-Boden-Gemeinschaften erstellt, welche zur Besiedlung der Reiswurzeln zur Verfügung standen. Dazu wurde eine inerte Sand-Vermiculit Matrix mit Reisfeldboden, bzw. Faulschlamm inokuliert und anschließend mit Reis bepflanzt. Die mikrobielle Aktivität der Boden-Systeme zur Bildung von Methan wurde in Vorversuchen getestet und die Besiedlung der Reis-Rhizosphäre daraufhin in bepflanzten Mikrokosmen untersucht. Diese enthielten jeweils eine unterschiedlich strukturierte mikrobielle Gemeinschaft, welche zur Wurzelbesiedlung zur Verfügung stand. Die Bedeutung der mikrobiellen Gemeinschaften für die Bildung von Methan aus pflanzenbürtigem Kohlenstoff wurde durch eine Kombination aus $^{13}CO_2$ pulse-labeling mit Illumina Sequenzierung und quantitativer PCR untersucht. Die molekularbiologischen Analysen richteten sich hierbei auf die 16S rRNA als phylogenetisches, bzw. *mcrA* und *pmoA* als funktionale Marker-Gene für methanogene Archaeen und methanotrophe Bakterien.

Die Bildung von Methan, sowie der generelle Abbau des wurzelbürtigen organischen Kohlenstoffs in den verschiedenen Mikrokosmen, war abhängig von der Zusammensetzung der bakteriellen und methanogenen Lebensgemeinschaften, jedoch nicht von deren absoluter Abundanz innerhalb der Rhizosphäre. Die Besiedlung der Rhizosphäre durch Bakterien und methanogene Archaeen fand in Abhängigkeit von den Ausgangs-Boden-Gemeinschaften statt, genauso wie die Translokation des photosynthetisch fixierten Kohlenstoffs. Dennoch zeigte sich anhand der an den Wurzeln vorkommenden mikrobiellen Zusammensetzung, dass die Rhizosphäre von Reispflanzen ein eigenständiges Habitat für Bakterien und methanogene Archaeen darstellt.

Es konnte eine methanogene Lebensgemeinschaft identifiziert werden, welche über die Rhizosphäre aller Mikrokosmen hinweg mit der Bildung von Methan aus Rhizodeposition verknüpft war. Neben den hydrogenotrophen *Methanocella* und *Methanobacteriaceae* konnten auch

Vertreter der acetoklastischen *Methanosaeta* dieser Gemeinschaft zugeordnet werden. Dennoch gehörten die meisten der Methanogenen, welche zur Methanemission aus Rhizodeposition beigetragen haben, zu denen mit hydrogenotrophem Stoffwechselweg. Innerhalb der methanogenen Gemeinschaft, die maßgeblich an der Bildung von Methan aus Rhizodeposition beteiligt war, wurden die Wurzeloberflächen stärker mit hydrogenotrophen Organismen besiedelt, während jene, die zur acetoklastischen Methanogenese befähigt sind, vornehmlich den Rhizosphären-Boden kolonisierten. Dies galt ebenfalls für die Besiedlung der Reis-Rhizosphäre durch Methanogene im Allgemeinen.

Darüber hinaus konnten Methanogene ermittelt werden, die ubiquitär in der Rhizosphäre aller Mikrokosmen vertreten waren. Diese wurden somit als methanogene Lebensgemeinschaft verstanden, welche Reispflanzen an ihren Wurzeln selektieren. Hierbei besiedelten Vertreter von *Methanobacteriaceae*, *Methanosaeta* und *Methanosarcina* die gesamte Rhizosphäre, während *Methanocella* im Rhizosphären-Boden aller Mikrokosmen vorhanden waren. Des Weiteren konnte gezeigt werden, dass alle methanogenen Vertreter, welche an der Bildung von Methan aus wurzelbürtigem Kohlenstoff beteiligt waren, dieser der Reiswurzel assoziierten Gemeinschaft angehörten. Somit war die an der Reiswurzel selektierte methanogene Lebensgemeinschaft auch an der Bildung von Methan aus pflanzenbürtigem Kohlenstoff beteiligt.

Neben methanogenen konnten auch bakterielle Gruppen identifiziert werden, die an der Entstehung von Methan aus Rhizodeposition beteiligt waren. Hierzu gehörten Vertreter von *Kineosporiaceae*, *Anaeromyxobacter*, *Bradyrhizobium* und *Bacteroidales*. Es konnte gezeigt werden, dass ein erhöhtes Vorkommen von *Kineosporiaceae* auch zu einer verstärkten Umsetzung von wurzelbürtigem Kohlenstoff zu Acetat, CO_2 und Propionat führte. Daher ist davon auszugehen, dass zumindest die Familie der *Kineosporiaceae* aktiv an der Umsetzung von Rhizodeposition zu Vorprodukten für die Methanogenese beteiligt war. Dennoch konnten keine Bakterien identifiziert werden, welche sowohl zur Bildung von Methan aus wurzelbürtigem Kohlenstoff beigetragen haben, als auch ubiquitär in der Rhizosphäre aller Boden-Systeme präsent waren. Dies könnte darin begründet liegen, dass die Zusammensetzung der mikrobiellen Gemeinschaft in der Rhizosphäre auch maßgeblich von der Verfügbarkeit bestimmter funktionaler Gruppen in den Ausgangs-Boden-Gemeinschaften abhängig war.

I. Introduction

1.1 Microbial colonization of the rice rhizosphere

1.1.1 The rice rhizosphere

Like for other vascular plants, the root of rice is the organ of the plant that typically lies below the soil surface. Rice plants develop a radical root which elongates to a length of about 15 cm. From this axis mesocotylic roots emerge between the node of the coleoptiles and the base of the radical. Therefore, the rice plants root system is basically composed of nodal roots, where tiller and roots arise simultaneously from the same node. Each node usually forms between 5 and 25 roots until maturity (Yoshida, 1982). The main functions of the rice roots are (i) absorption of water and inorganic nutrients, (ii) anchoring the plant-body to the soil, (iii) supporting the storage of nutrients and assimilates of photosynthesis, (iv) vegetative reproduction, and (v) competition with other plants.

Since rice plants grow in soil, they are placing their roots in direct proximity to a high abundance of microbial diversity (Tringe et al., 2005), making the roots one of the favorable habitats for colonization by soil microorganisms (Kimura et al., 1988). Therefore, roots are the primary site of direct interaction between plants and soil microorganisms (Sessitsch et al., 2011). Furthermore, the plants impact extends to the area of the soil which is surrounding the root. This part of the soil, where microorganism-mediated processes are under the influence of the plant root, is defined as the rhizosphere (Hiltner, 1904). Further is the influence of the plant root to the soil and the soil microorganisms declared as rhizosphere effect (Curl and Truelove, 1986). The rhizosphere is divided into the sections of, (i) root surrounding soil (ectorhizosphere); (ii) the root surface (rhizoplane); and (iii) the different cell layers of the root (endorhizosphere) (Lynch and Whipps, 1990).

All these sections are locations of intense plant-microbe-, as well as microbe-microbe-interactions. In the rhizosphere, plants have the ability to mediate both positive and negative interactions with soil microorganisms (Bais et al., 2006). Positive interactions include several symbiotic factors, like

utilization of plant released carbon by microorganisms (Bais et al., 2006; Dennis et al., 2010), biocontrol of plant-pathogens, availability of micronutrients, enhancement of plants stress tolerance, release of phytohormones (Berg and Smalla, 2009), and a positive effect on the plants immune-system (Jones and Dangl, 2006), while plant pathogenesis is the negative site of interactions (Berg and Smalla, 2009).

Through the influence by the rice root are physical, biological, and chemical conditions of the whole rhizosphere different from the surrounding anoxic bulk soil (Barea et al., 2005; Manoharachary and Mukerji, 2006), distinctly separating this two compartments in a rice field (Liesack et al., 2000; Conrad, 2007). The aerenchyma of rice plants facilitates the transport of oxygen to the roots (Armstrong, 1967; Hoshikawa et al., 1993). The primary function of this root aeration is to provide O_2 for aerobic processes within the plant tissue. Thereby, some O_2 is released into the rhizosphere (Armstrong, 1967). But for rice plants, this radial oxygen loss (ROL) by the root is a dynamic phenomenon. A growing root shows an oxic influence extending up to several millimeters behind the root tip, while at older parts of the root the redox potential becomes reducing again (Flessa and Fischer, 1992). The volume of the root surrounding oxic zone is influenced by root permeability, root-, and soil respiration (Bosse and Frenzel, 1997). Through its positive redox potential, molecular oxygen is one of the most important reactants in biogeochemical cycles (Brune et al., 2000).

Maybe even more important is the interaction of the rice plant with the root associated microorganism, by the release of potentially valuable small molecular weight compounds - the root exudates (Bais et al., 2006). Those compounds include many carbon containing molecules like organic acids, sugars, amino acids, aliphatic compounds, aromatics, proteins, enzymes, lipids, coumarins, flavonoids (Berg and Smalla, 2009), siderophores, as well as signal molecules (Pinton et al., 2001), and lead to a high microbial activity in the rhizosphere (Barea et al., 2005; Manoharachary and Mukerji, 2006). Soil microorganisms choose the rhizosphere as a habitat because roots are an abundant source of organic substrates (Curl and Truelove, 1986; Kimura et al., 1988). Compounds released by the root play a role in organismic interactions and can be classified into nutrients, foods, and allelochemicals (Whittaker and Feeny, 1971). The main sites for exudation are root tips, root hairs, and regions of lateral root development (Curl and Truelove, 1986). However, amount and composition of root exudates is specific for each plant family or species (Berg and Smalla, 2009). Many factors like light intensity, temperature, nutritional status of the plant, stress factors, mechanical impedance, soil type, plant age, and microbial activity have a

clear influence on their release (Pinton et al., 2001). Root exudates are important for chelation and solubilisation of nutrients in soil aggregations and are further effecting the pH-value of the rhizosphere (Hale and Moore, 1980).

Beside exudates, the plant root also releases additional carbon compounds, like lysates, mucilage, secretions, dead cell material, and gases – including respiratory CO_2 (Lynch and Whipps, 1990). The total amount of transferred carbon from the plant roots into the rhizosphere is defined as rhizodeposition (Pinton et al., 2001; Singh et al., 2004). Rhizodeposits can reach the rhizosphere in a passive way by diffusion or autolysis of dead cells as diffusates, or are actively excreted as secretions (Pinton et al., 2001). The plant is continually producing and excreting carbon compounds via the root, thus rhizodeposits represent a key factor for the enrichment of specific microbial populations in the rhizosphere (Uren, 2001). Since the composition of root exudates differs between different plant species, each species is thought to select a specific microbial root community as their root microbiome (Kowalchuk et al., 2002; Bais et al., 2006). Exudation patterns are also different between particular rice cultivars and even between the different growth stages of the rice plant (Aulakh et al., 2001). Therefore, the type of the rice cultivar also plays a role for colonization of the rhizosphere (Conrad et al., 2008) and further, the bacterial root community also changes with the plants grow stage (Ikenaga et al., 2003). By release of root exudates plants have a direct influence on the microbial community composition in the vicinity of their roots (Somers et al., 2004). But this effect seems to be constricted to the rhizosphere. The impact of a single plant species is thought to reduce the bacterial diversity in a soil, while a greater variety of different plant species maintains a higher level of bacterial diversity (Kowalchuk et al., 2002).

In general, microorganisms are colonizing the rhizosphere of rice in several ways. Beside the rhizospheric soil, many microorganisms are living in the mucigel layer in the rhizoplane (Jenny and Grossenbacher, 1963; Greaves and Darbyshire, 1972), or as endophytes inside the rice roots without causing any apparent harm to the host (Hardoim et al., 2012). However, a high degree of plant specificity for general microbial colonization of roots is for sure (Berg and Smalla, 2009).

1.1.2 Colonization of rice roots by methanogenic archaea

Beside many other groups of microorganisms, rice roots are colonized by fermenting bacteria (Ikenaga et al., 2003), as well as methanogenic archaea (Großkopf et al., 1998b), since the microbial community of rice paddy soil is apparently a sufficient seed bank for colonization by methanogenic species (Pump et al., 2015). Nevertheless, methanogenic archaea as endophytes

are still absent in rice plants (Sessitsch et al., 2011). The methanogenic archaea are a large group within the phylum of Euryarchaeota, which form methane as the major product of their energy metabolism (Whitman et al., 2006) and are divided into the seven orders: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales* (Narihiro and Sekiguchi, 2011), and *Methanomassiliicoccales* (Borrel et al., 2014).

Microbial degradation of organic matter to methane and carbon dioxide is a common process under anaerobic conditions, as in flooded rice paddy soils (Conrad, 1997; Conrad, 2009; Schink and Stams, 2013). This reaction is not performed by a single group of microorganisms but by a syntrophic microbial community (Thauer, 1998). In the whole process, the methanogenic archaea play a key role at the end of the anaerobic degradation of organic compounds (Liu and Whitman, 2008). Methanogenesis is the typical terminal electron-accepting-process when other acceptors like oxygen, nitrate, sulfate, or oxidized iron and manganese species have already been reduced. These reduction processes follow a specified sequence, which is determined by thermodynamics. That implies, compounds with a higher redox potential are reduced first (Ponnamperuma, 1972) **(Figure 1.1)**. Although, methanogens are described as strict anaerobes (Whitman et al., 2006), these archaea perform methanogenesis in the rice rhizosphere, as well as directly on the root surface, despite the oxic influence of the rice roots (Großkopf et al., 1998b; Chin et al., 2004; Lu and Conrad, 2005; Conrad et al., 2008).

The first step of the syntrophic organic matter degradation is the hydrolysis of polymers (polysaccharides, proteins, nucleic acids, and lipids) to oligo- and monomers (sugars, amino acids, purines, pyrimidines, fatty acids, and glycerol) (Schink and Stams, 2013). This step is followed by fermentation of monomers to fatty acids, alcohols, acetate, hydrogen, and carbon dioxide. Fermentation products such as acetate, hydrogen, carbon dioxide, and other one-carbon compounds, can be used directly by methanogens for conversion to methane and carbon dioxide (Conrad, 2002; Schink and Stams, 2013). But also other fermentation products, e.g. short chain fatty acids (longer than two carbon atoms), alcohols (longer than one carbon atom), branched-chain and aromatic fatty acids can be used after secondary fermentation by other groups of bacteria (Schink and Stams, 2013). With secondary fermentation these substrates are also converted to acetate, carbon dioxide, hydrogen, and perhaps also formate, which subsequently can be used by the methanogens (Conrad, 2002; Schink and Stams, 2013).

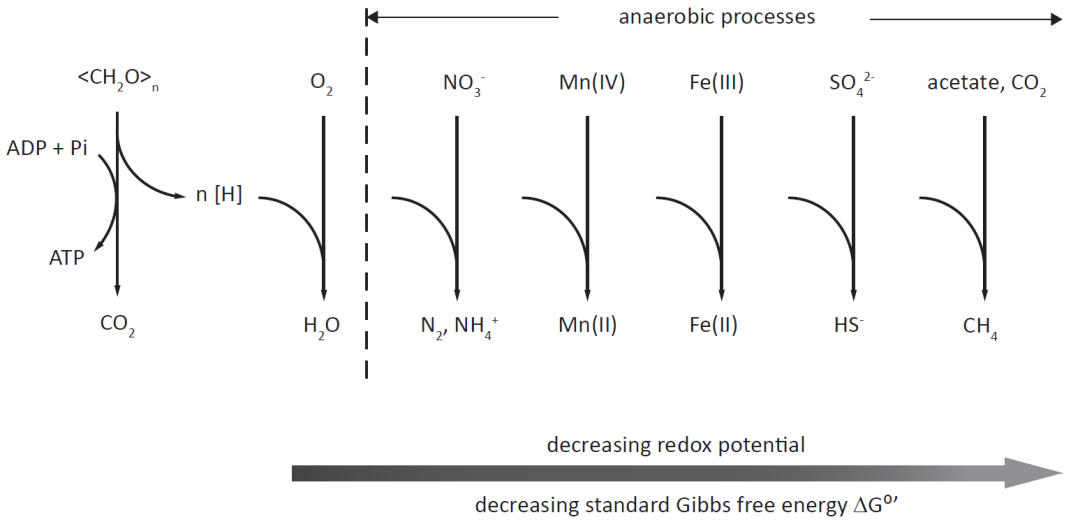

Figure 1.1 | Sequence of microbial reduction processes coupled to oxidation of organic matter in rice paddy soils. After (Ponnamperuma, 1972).

Despite their phylogenetic diversity, methanogenic archaea are limited in utilizable substrates for formation of methane. Methanogenic activities are often classified into the three pathways of, hydrogenotrophic reduction (hydrogenotrophic methanogenesis), acetate cleavage (acetoclastic methanogenesis), and conversion of methylated C1 compounds **(Table 1.1)**. Hydrogenotrophic methanogens usually use carbon dioxide as an electron-acceptor, while hydrogen serves as electron-donor. Since carbon dioxide is usually abundant in anaerobic environments, hydrogen concentration maybe the limiting factor for this reaction. Some hydrogenotrophic methanogens can also use formate as major electron-donor, whereas others can use secondary alcohols or ethanol. However, methanogenesis based on hydrogen and carbon dioxide is common among most methanogenic archaea. Carbon dioxide is reduced successively through formyl, methylene and methyl-levels to methane (Liu and Whitman, 2008).

Acetate is a major intermediate in anaerobic degradation and also can be used as substrate for methanogenesis. For this pathway, acetate is cleaved by oxidizing the carboxyl group to carbon dioxide, and reducing the methyl-group to methane. Acetoclastic methanogenesis is performed by the genera of *Methanosarcina* and *Methanosaeta*, for the latter this pathway is obligatory (Liu and Whitman, 2008).

Table 1.1 | Standard Gibbs free energy changes ($\Delta G^{0'}$) of typical methanogenic reactions. After (Conrad and Frenzel, 2002; Hedderich and Whitman, 2006).

reaction	$\Delta G^{0'}$ (kJ/mol CH_4)
hydrogentrophic	
$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	-131
acetoclastic	
$CH_3COOH \rightarrow CH_4 + CO_2$	-33
conversion of methylated C1 compounds	
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	-103

To perform the different complex methane generating pathways several key enzymes and coenzymes are necessary. These enzymes and coenzymes are shared by all methanogens. Biochemical components for methanogenesis through hydrogenotrophic CO_2 reduction and conversion of methylated C1 compounds are nearly the same. These pathways are somewhat different from those of acetoclastic methanogenesis, but all share the coenzymes tetrahydromethanopterin (H_4MPT) and F_{420}-hydrogenase – even if H_4MPT is used to bind different functional groups within the different pathways. However, the reduction of the methyl-group, bound to the methyl-coenzyme M (CH_3-S-CoM) by a hydrogen bound, is the last step of methanogenesis and identical in all pathways. This final reaction is catalyzed by the methyl-coenzyme M reductase (Thauer, 1998), which is homologous for all methanogenic archaea. The *mcr* gene, which codes for the methyl-coenzyme M reductase - or, to be more precise, its small α subunit (*mcrA*) - is relatively well conserved and common for all methanogens. Hence, the *mcrA* gene is a suitable functional genetic marker for the identification of methanogenic archaea in several environments (Luton et al., 2002; Friedrich, 2005).

Conditions for methanogenic archaea in the rhizosphere are different compared to the anaerobic rice paddy soil. Hence, methanogens have to face a different environment, as well as many interactions with other microorganisms and also the plant itself, especially through the oxygen loss of the roots. If an anoxic environment, colonized by methanogenic archaea is exposed to oxygen the transcription of mcrA is decreased (Yuan et al., 2009), which is resulting in reduction of methanogenic activity (Yuan et al., 2009; Yuan et al., 2011). Nevertheless, the methanogenic community structure is not changed by the exposure to oxygen (Yuan et al., 2009). Furthermore, many methanogens are neither very sensitive to high redox potential, nor to the presence of oxygen (Fetzer et al., 1993; Fetzer and Conrad, 1993). For species of *Methanocella* it could be shown that they posses enzymes involved in oxygen detoxification (Erkel et al., 2006).

However, since also fermenting bacteria are colonizing the rice rhizosphere (Ikenaga et al., 2003), rice plants possess a microbial root community which is able to perform the complete, syntrophic degradation of root derived carbon right up to methane (Conrad and Klose, 2000). Regardless of where in the paddy soil the methane is formed, directly after production the methane may be transported to the atmosphere via the aerenchyma tissue of the rice plant (Nouchi et al., 1990; Minoda and Kimura, 1994). Therefore, rice fields are an important anthropogenic source for atmospheric methane, which contribute about 10 % to the global methane cycle (Conrad, 2009).

1.1.3 Plant vs. soil – effects on colonization of rice roots and emission of methane

Beside the plant, also several other factors are influencing the microbial community in the rice rhizosphere. Edaphic variables, especially pH-value (Nunan et al., 2005; Fierer and Jackson, 2006), soil type (Girvan et al., 2003; Fierer and Jackson, 2006), and soil structure (Siciliano et al., 2001; Nunan et al., 2005) have an influence on the structural and functional diversity of microbial communities in the rhizosphere. Therefore, also the composition of the methanogenic community in the rhizosphere is influenced by soil factors (Conrad et al., 2008; Pump et al., 2015). There are several contrary opinions which are suggest soil type or plant species as dominant factor for determining the root microbiome (Graystone et al., 1998; Girvan et al., 2003; Nunan et al., 2005). It is for sure that soil properties, as well as the plant species influences the structure and function of the microbial community, but the extent to which both factors contribute to microbial communities is not fully understood (Kowalchuk et al., 2002; Berg and Smalla, 2009). Furthermore, even if the functional diversity of microorganisms in different soils may be similar, it could be a combination of environmental and plant factors affecting the activity and proliferation of microorganisms under different conditions (Graystone et al., 1998).

In general, the most important factors influencing methane production in rice paddy soils include soil type, rice variety, temperature, soil redox potential, and the amount of organic carbon and nitrogen (Conrad, 2002). In combination with rice plants, carbon assimilation and translocation processes, as well as microbial abundance in the rhizosphere are affected by plant and soil and therefore both have an impact on the emission of methane (Conrad et al., 2008; Pump et al., 2015), since production of methane is stimulated by the release of root derived carbon compounds (Dannenberg and Conrad, 1999). However, to what extent rhizodeposition, the primordial microbial soil community and abiotic soil factors contribute to the colonization of rice roots by methanogenic archaea and the emission of methane is not fully understood. Nevertheless, composition of the methanogenic community colonizing rice roots has a potentially important impact on the global methane cycle (Conrad et al., 2008).

1.2 Formation of methane from root derived carbon

On average, 30 - 60 % of the plants net photosynthesized fixed carbon is allocated to the root, while 40 - 90 % of this fraction enters the soil as rhizodeposition (Pinton et al., 2001). Large amounts of root derived carbon are consumed by microorganisms in the rhizosphere (Hernández et al., 2015), whereas on average 0.54 % of the photosynthetic assimilated carbon is incorporated into microbial biomass over the growing season of rice plants (Lu et al., 2002). Since plant derived carbon is the most important carbon source for emission of methane from rice field soils (Lu and Conrad, 2005), production of methane in the rhizosphere is even higher compared to the anoxic bulk soil (Lu et al., 2000). Up to 60 % of emitted methane is produced from root derived carbon, while the rest is formed by degradation of rice straw and soil organic matter (Yuan et al., 2012). Since direct contribution of photosynthesized CO_2-carbon becomes smaller during later vegetative stages of rice growth, it is thought that subsequently partially decomposed root tissues might be a more important source for CH_4 at this state (Minoda and Kimura, 1994).

Rice roots release several carbon compounds, which serve as readily available substrates for the microbial degradation to methane (Dannenberg and Conrad, 1999; Lu et al., 2005; Pump and Conrad, 2014). This includes secretion of several amino acids, sugars like fructose, glucose, sucrose (Lin and You, 1989), as well organic acids like formic, acetic, tartaric, oxalic (Aulakh et al., 2001), lactic, malic, succinic and citric acid (Lin and You, 1989; Aulakh et al., 2001). But also carbohydrates in form of cellulose, hemicellulose, and lignin are derived from the plant root (Conrad, 2007). A full cycle from the assimilation of CO_2 by the plant to the emission of CH_4 - via

the release of rhizodeposition through the plant root - takes about two hours (Megonigal and Guenther, 2008). Following this cycle by labeling of CO_2 revealed that photosynthesized carbon is translocated to the rice roots and from there released into the rhizosphere, where it is converted to CH_4 (Minoda and Kimura, 1994; Dannenberg and Conrad, 1999; Pump and Conrad, 2014). Acetate is thought to be the major fermentation product on rice roots, while besides hydrogen also minor amounts of propionate, butyrate, valerate and isovalerate are formed by fermentation (Conrad and Klose, 2000). Furthermore, the conversion of root released carbon is thought to start from hexose via lactate, propionate and acetate to methane (Dannenberg and Conrad, 1999), while also degradation of the formed propionate to acetate, carbon dioxide, and hydrogen occurred through the succinate pathway by fermenting bacteria (Krylova et al., 1997).

Although fermentation pattern of root-released carbon apparently is not much different from that of rice straw and soil organic matter, the microbial community involved seems to be completely different (Conrad and Frenzel, 2002). Different bacterial groups are involved in the degradation of root derived carbon in the rhizospheric soil compared to the rice root surface, reflecting the different physio-chemical characteristics in these compartments. Further, it is thought that the microbial community directly colonizing the root surface is more involved in fermentation of root derived carbon compared to that of the ectorhizosphere (Hernández et al., 2015). This may be important, since methanogenesis takes place at the end of the syntrophic degradation of organic matter, and therefore methanogenic archaea are dependent on further degradation steps and fermentation products.

Also the composition of the methanogenic community in the rhizosphere is different from the bulk soil (Großkopf et al., 1998b). The genera of *Methanocella* and *Methanosarcina* seem to be more active on rice roots than other methanogenic archaea (Lu et al., 2005; Lu and Conrad, 2005). Since H_2/CO_2 is thought to be the main substrate of CH_4 production on rice roots (Conrad and Klose, 2000; Chin et al., 2004), methanogens colonizing the rice root are assumed to produce CH_4 mainly via hydrogenotrophic methanogenesis (Conrad, 2007), in contrast to the bulk soil where about 33 % of the CH_4 is formed by the hydrogenotrophic pathway (Conrad, 1999). An important factor for colonization of roots by hydrogenotrophic methanogens seems to be the availability of H_2, since a generally low H_2 partial pressure in rice field soil could be a limiting factor for hydrogenotrophic methanogenesis (Conrad, 2007). However, even if the rice rhizosphere is dominated by hydrogenotrophic methanogens, also the type of that organisms matter for ecological function, even if they belong to the same functional group (Conrad et al., 2008).

Since acetate is a primary fermentation product for degradation of rhizodeposits (Conrad and Klose, 2000), acetate can reach millimolar concentrations in the root surrounding area (Conrad, 2007). This could be the reason why *Methanosarcina* are more dominant at rice roots than *Methanosaeta*, since *Methanosarcina* are thought to be more efficient in that habitat for performing acetoclastic methanogenesis compared to *Methanosaeta* (Chin et al., 2004; Lu and Conrad, 2005).

Composition of the methanogenic community not only differs between the rhizosphere and the bulk soil, but also the methanogenic community of the rhizospheric soil is further different from that directly on the root surface. However, all parts of the rhizosphere contain members of most of the major methanogenic orders and families (Watanabe et al., 2010; Pump et al., 2015). By providing rhizodeposition to the rhizosphere, rice plants show a direct influence on the formation of CH_4 (Conrad, 2007). Further, is the emission of methane affected by the compositions of root colonizing microbial communities (Conrad et al., 2008). However, the colonization process of rice roots by microorganisms involved in degradation of rhizodeposits to methane as a whole is still ambiguous. Nevertheless, is it thought that rates of photosynthesis-driven CH_4 emission are limited by the abundance of methanogens on the roots (Pump and Conrad, 2014, Pump et al., 2015).

1.3 Rhizosphere interactions linked to the emission of methane

1.3.1 Methane-oxidation by methane-oxidizing bacteria

Not all of the produced methane is actually emitted to the atmosphere (Frenzel, 2000). The emission of methane can be assumed as the balance between methanogenic activity and methane oxidation (Gilbert and Frenzel, 1998). About 20 % of produced methane is directly consumed by methane-oxidizing bacteria (MOB), colonizing the rhizosphere of rice plants (Frenzel, 2000). These methanotrophs are a subgroup of the methylotrophs, and are generally limited to use methane and methanol as their sole carbon- and energy source. Methanotrophic bacteria are divided into the groups of type I and type II (Bowman, 2006). Type I methanotrophs belong to the γ-proteobacteria (Hanson and Hanson, 1996) and perform carbon assimilation by the ribulose monophosphate pathway (RuMP), while type II methanotrophs belong to the α-proteobacteria (Bowman, 2006) and use the serine cycle for carbon assimilation (Trotsenko and Murrell, 2008). The aerobic methanotrophs oxidize methane via the intermediates methanol, formaldehyde, and formate up to carbon dioxide. The unique enzyme for the methanotrophs is the methane monooxygenase (MMO), catalyzing the conversion of methane to methanol (Hanson and Hanson, 1996). The *pmoA* gene encodes a subunit of the particular methane monooxygenase (pMMO) and is the mostly used

functional marker gene for targeting methanotrophs (McDonald et al., 2008; Dumont et al., 2014). However, in the vast majority of terrestrial ecosystems aerobic methane oxidation is just taking place in aerobic habitats (Conrad, 1996; Conrad, 1997), like the rice rhizosphere (Frenzel, 2000). Hence, the rice rhizosphere is been supplied with methane as well as oxygen, the rhizosphere is an ecological niche for methane-oxidizing bacteria, which can be metabolic active in this area of the soil (Gilbert and Frenzel, 1998). Indeed is the abundance of methanotrophs usually higher in the rhizosphere compared to the bulk soil (Conrad, 2007). Contrary to the methanogenic archaea, methane-oxidizing bacteria can live endophytic in rice roots (Hardoim et al., 2012), or even colonize the xylem vessels of the rice plants (Gilbert et al., 1998).

Emission of methane may be significantly reduced by the activity of methane-oxidizing bacteria in the rhizosphere. As a result, soils densely rooted by rice plants show a low concentration of methane (Gilbert and Frenzel, 1998). It is assumed that 20 % of the global microbial oxidation of methane depends on the interaction of methanotrophs with wetland plants (Frenzel, 2000). But compared with other aerobic environments, the methane-oxidation in the rice rhizosphere seems to be ineffective. The reason for this may be the competition for nutrients between rhizospheric bacteria and rice plants (Conrad and Frenzel, 2002), since potential methane-oxidation has a positive correlation with availability of ammonium (Krüger et al., 2001). The community of methane-oxidizing bacteria, colonizing the rice rhizosphere is highly diverse and consists of both type I and type II methanotrophs (Horz et al., 2001). Which plant factors are controlling the oxidation of methane in soils is still ambiguous. Nevertheless, is it thought that availability of nitrogen (Conrad, 2007), as well as local oxygen-, (Van Bodegom et al., 2001) and methane concentrations (Gilbert and Frenzel, 1998) are important. Furthermore, methanotrophs are thought to be only able to outcompete heterotrophs at low oxygen concentrations. This means methane-oxidation rates in the rice rhizosphere are highly variable (Van Bodegom et al., 2001).

Beside the aerobic methane-oxidation, methane can be oxidized under anaerobic conditions, coupled to reduction of nitrite (Raghoebarsing et al., 2006), nitrate (Haroon et al., 2013), sulfate (Hoehler et al., 1994), iron or manganese (Beal et al., 2009) instead of oxygen. While sulfate, iron, and manganese dependent methane-oxidation mainly occurs in marine sediments (Valentine and Reeburgh, 2000; Beal et al., 2009), nitrate dependent methane oxidation could not be assigned to a natural environment so far (Haroon et al., 2013). Nitrite driven methane-oxidation on the other hand also takes place in paddy soils (Hu et al., 2014). If anaerobic oxidation of methane also plays a role for the rice rhizosphere is not clear, since methanotrophs involved in nitrite dependent methane-oxidation in paddy soils showed a low abundance and potential methane-oxidation rate in presence of oxygen (Luesken et al., 2012).

However, microbial interaction between methanogenic archaea and methane-oxidizing bacteria can also occur in contrary direction. Carbon dioxide, originating from oxidation of methane can serve again as a precursor for hydrogenotrophic methanogenesis (Dannenberg and Conrad, 1999).

1.3.2 Microbial-interactions of methanogens with other redox-cycles

Not all bacteria in the rhizosphere are involved in the formation of methane or belong to the methane-oxidizing bacteria. Several microbial processes in the rhizosphere, involved in different redox-cycles, are influenced by the rice root and further show an effect on the emission of methane. Since, methanogenesis is an energetically unfavorable process, production of methane is suppressed while other electron-acceptors are reduced (Conrad and Frenzel, 2002; Conrad, 2007). However, the nitrate-, sulfate- and ferric iron reducers form functionally and taxonomically diverse communities by themselves (Conrad, 2007). But also for these groups the rhizospheric communities seem to be different from that of the anoxic bulk soil (Lüdemann et al., 2000; Noll et al., 2005).

Methanogenic archaea in the rhizosphere underlie several interactions with the rice plant, as well as with microorganisms of other, different redox-cycles **(Figure 1.2)**. Using an energetically unfavorable process at the end of the degradation of organic matter, methanogens have to compete for acetate and hydrogen with other microorganisms that use other electron-acceptors more effectively (Chidthaisong and Conrad, 2000). Analogously to CH_4, the reduced forms of these inorganic electron-acceptors can be oxidized under the presence of O_2 (Conrad and Frenzel, 2002). This becomes even more important in the rhizosphere, because due to the presence of the root, nitrate, Fe(III), and sulfate are regenerated by oxidation with O_2 from their reduced forms (Brune et al., 2000; Liesack et al., 2000; Conrad and Frenzel, 2002). These regenerated oxidants can subsequently serve again as electron-acceptors for reduction (Conrad and Frenzel, 2002), and therefore methanogens have to compete for substrates again (Inubushi et al., 1984; Yao et al., 1999).

The very high O_2 sensitivity of Fe(II) at neutral pH enables its oxidation at soil locations with a high root density, which leads to high concentrations of Fe(III) (Liesack et al., 2000). Therefore, Fe(III) concentrations in the rice rhizosphere are almost twice as high compared to unplanted paddy soil (Ratering and Schnell, 2000). It is thought that Fe(III) has a direct inhibitory effect on methanogenic archaea by inhibiting methanogenesis, especially the hydrogenotrophic pathway (van Bodegom et

al., 2004). Like methanogenic archaea, iron-reducing bacteria are also known to colonize the rice root (Scheid et al., 2004). But some methanogens are also able to reduce Fe(III) to Fe(II) instead of CO_2 to CH_4 (van Bodegom et al., 2004).

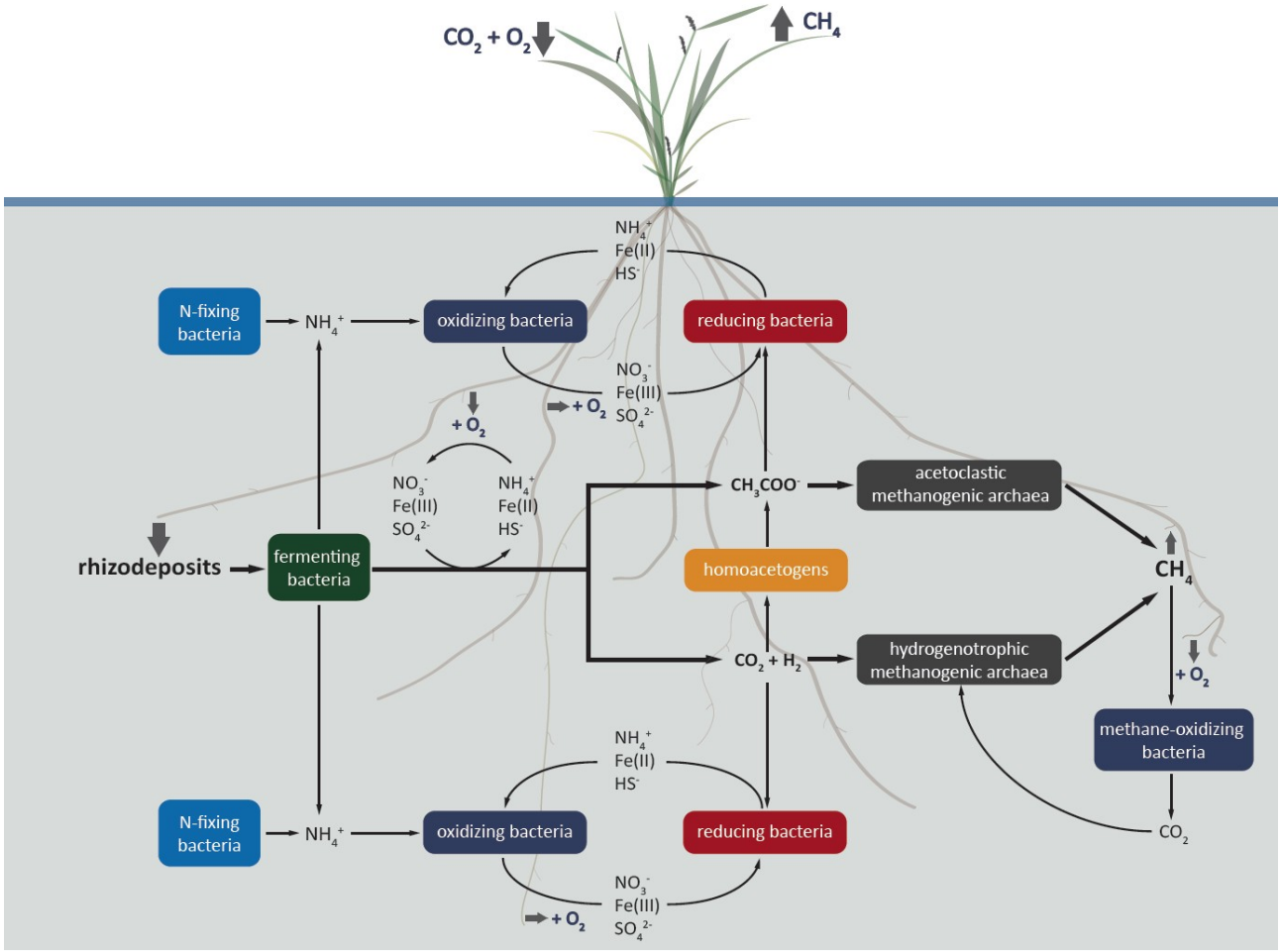

Figure 1.2 | Simplified scheme of interactions of methanogenic archaea with microorganisms related to other redox-cycles in the rice rhizosphere. Modified from (Conrad and Frenzel, 2002) and (Conrad, 2007).

Ammonium can undergo nitrification in the rhizosphere of rice plants (Liesack et al., 2000). Some of the so formed nitrate can subsequently be reduced or is be assimilated by the rice plant. However, high rates of coupled nitrification and denitrification take place in the rhizosphere (Reddy et al., 1989). Presence of reducible nitrogen compounds (NO_3^-, NO_2^-, NO, N_2O) are important factors for the formation of CH_4 (Conrad, 2002). Like Fe(III), also presence of these N-compounds cause a suppression of methanogenic activity on rice roots (Scheid et al., 2003). Reason for this is that, beside higher efficiency for utilization of acetate and H_2 by nitrate-reducing bacteria, also nitrate-reduction may be coupled to oxidation of glucose. Therefore, the competition of nitrate reducers with fermenting bacteria for organic compounds also lowers the amount of acetate and H_2, which then cannot be used by methanogenic archaea (Chidthaisong and Conrad, 2000). Furthermore is the denitrification in the rhizosphere dependent on nitrate, originating from oxidation of ammonium. Since plants also use ammonium as a nutrient, nitrate-oxidizing bacteria are in direct competition with the plants for there substrate (Verhagen et al., 1995).

With the presence of sulfate in the rhizosphere, also sulfate-reducers can outcompete methanogenic archaea for acetate and H_2 (Conrad, 2007). Re-oxidation of sulfate is a process taking place in the rhizosphere, since sulfate concentrations are increasing with vicinity to the roots (Wind and Conrad, 1997). Also, sulfur-reducing and sulfur-oxidizing bacteria are known to colonize rice roots (Scheid and Stubner, 2001). Like for Fe(III) and nitrate, also the activity of sulfate-reducers can suppress methanogenesis in the rhizosphere (Scheid et al., 2004).

However, several rhizospheric cycling processes, linked to formation of methane, are effected by release of rhizodeposition and O_2 from the rice plant (Conrad and Frenzel, 2002). Nevertheless play methanogens an important role in the rhizosphere of rice. Interestingly can some methanogens regain their activity faster after exposure to oxygen than sulfate- and Fe(III)-reducers when competing for H_2 (Lueders and Friedrich, 2002). Beside enzymes for oxygen detoxification, it has been shown that species of *Methanocella* posses enzymes involved in carbohydrate metabolism, as well as assimilation of sulfate and nitrogen compounds (Erkel et al., 2006). All these characteristics can be an advantage for growth in the rhizosphere, where oxygen and sulfate concentrations may be temporarily high, nitrogen may be limiting and organic substrates abundant and diverse (Conrad, 2004).

1.4 Aims of this study

Methane from rice paddy soils contributes about 10 % of the total global emission of methane. The rhizosphere represents an interface for many of plant-microbe interactions involved in the formation of methane by the degradation of rhizodeposits. Since plants have a clear influence on the microbial soil community via the release of root derived carbon, it is thought that rice plants shape their distinct microbial community in the rhizosphere. Each variant of a soil possesses an individual microbial community, which can serve as a seed bank for the colonization of rice roots planted in the soil. Although some research is focusing on the colonization of rice roots by methanogenic archaea, little is known about the rice root microbiome with respect to this initial community of a soil available for colonization. While microbial diversity and activity in the rhizosphere clearly have an effect on the formation of methane, the link between the rhizospheric community and the production of methane by the degradation of root derived carbon compounds is still ambiguous.

Therefore we used new experimental tools like assembling synthetic initial soil communities, pulse-labeling, and illumina sequencing to address the following questions:

I. What are the similarities and differences in microbial colonization of the rice rhizosphere with respect to different initial communities from certain environments?

Synthetic microbial communities have been created by the combination of an inert and sterile soil matrix with different environmental samples, which contain variations of microbial communities that are able to degrade organic compounds to methane. In order to create viable plant-soil model-systems, a former approach from (Pump and Conrad, 2014) was modified and tested with regard to efficiency and the potential emission of methane. This created synthetic soil-systems served as a seed bank for the colonization of rice roots in plant-soil microcosms. We assessed the diversity of bacteria and methanogenic archaea to gain an insight into the composition of the community in the rhizosphere of rice plants with respect to the different initial communities.

II. What is the impact of the root microbiome on carbon translocation and degradation of root derived carbon in the rice rhizosphere?

Rice planted-soil microcosms with synthetic soil-systems have been pulse-labeled with $^{13}CO_2$. Carbon translocation was followed from photosynthetic fixation by the plant through the degradation of root released carbon compounds up to precursors for methanogenesis. Bacterial diversity and activity was investigated further in order to determine active microbial processes which could be linked to the degradation of root derived carbon compounds.

III. In how far is the microbial colonization of rice roots of concern to the formation of methane from root derived carbon?

Carbon translocation in plant-soil microcosms was further followed up until the formation of methane from root derived carbon compounds. Diversity and activity of the methanogenic archaea and methane-oxidizing bacteria were determined in order to link the microbial community composition of the rice rhizosphere to the emission of methane originating from rhizodeposition.

II. Material and methods

2.1 Chemicals, gases and filters

Unless otherwise noted, all chemicals were purchased at a quality level “for analysis” from the following suppliers: Fluka (Buchs, Switzerland), IDL GmbH & Co KG (Nidderau, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Karlsruhe, Germany), and Stähler Internetional GmbH & Co. KG (Stade, Germany). Molecular reagents, enzymes, oligonucleotides and reaction tubes were received from Kapa Biosystems (Boston, United States), Life Technologies GmbH (Darmstadt, Germany), Macherey-Nagel (Düren, Germany), Promega GmbH (Mannheim, Germany), and Sigma-Aldrich (Karlsruhe, Germany). Gases were purchased from Air Liquide (Düsseldorf, Germany), Campro Scientific (Berlin, Germany), Euriso-Top (Saarbrücken, Germany), ICI Chemicals and Polymers Ltd. (Runcorn, United Kingdom) and Messer Industriegase GmbH (Siegen, Germany). Materials for microcosms were purchased from Roth (Karlsruhe, Germany) and Thinex New Media (Dortmund, Germany), and sampling equipment from Rhizosphere research products (Wageningen, Netherlands), Terumo Deutschland GmbH (Düsseldorf, Germany), and MAGV GmbH (Rabenau-Londorf, Germany).

2.2 Environmental samples and sampling procedures

For this study we used rice field soil from the „Instituto Sperimentale della Cerealicoltora” in Vercelli, Italy. This paddy soil is well established and previously used for many experiments with plant-soil microcosms. A second paddy soil was sampled from a rice field located at the International Rice Research Institute (IRRI) in Los Banos, Philippines. Digested sludge was originated from the municipal sewage treatment plant of Giessen, Germany. Digested sludge is the final product of clarification and debris of an anaerobic methane digester. After digestion, the sludge was dried in a chamber filter press, as well as by centrifugation. Further, soil- and digested

sludge samples were dried at room temperature, shredded, and sieved through a stainless steel screen (2 mm mesh).

The straw used in this study was originated from another rice field than the soil samples, since the microbial soil community involved in degradation of organic matter may be adapted to the specific composition of the plant material when originated from the same sampling site. Therefore, we used rice straw from rice plants harvested in the year 2009 at a Chinese rice field near Cixi, in the province of Zhejiang (30°23'N, 120°17'E). Furthermore, field-grown rice plants have been used instead of plants grown in a greenhouse to maintain straw composition in a natural state. After harvesting, the rice plants were dried on the field, stored at room temperature, and milled to a particle size smaller than 1 mm.

2.3 Synthetic soil model-system

Environmental samples from rice paddy soils and digested sludge were used as origin of different initial microbial communities for colonization of the rice roots. Since the samples have different properties it was necessary to minimize the different physical and chemicals parameters. Therefore, a synthetic soil model-system (Pump and Conrad, 2014) was modified to reach a sufficient root colonization, as well as emission of methane. Synthetic microbial communities were created in artificial soil-systems by combination of 20 % (m/m) environmental sample as inoculum and 80 % of an inert soil-matrix (m/m, with 79 % sand and 1 % vermiculite). For assembling of the inert matrix we used quartz-sand (crystalline, 0.4-0.8 mm, Roth) and vermiculite (fine, 0-1 mm, Thinex New Media). As inocula served, (i) Vercelli rice paddy soil, (ii) digested sludge, (iii) a mixture of digested sludge and Vercelli soil (1:1), and (iv) Philippine rice paddy soil.

To use this artificial soil-systems in the plant-soil microcosms and simultaneously ensure a sufficient colonization of the roots by methanogenic archaea, the initial methanogenic community has to been stimulated. One strategy for stimulation was the addition of rice straw to the artificial soil-systems, which is known to enhance the production and emission of methane (Kimura et al., 2004). To obtain the natural composition of the initial microbial communities, rice straw had to been sterilized before addition. Different ways of sterilization were tested and therefore the straw was treated with heat sterilization, autoclaving, or γ-radiation. For heat sterilization straw samples of 5 g were placed in aluminum cups and exposed to 140°C for 6, 12 or 24 hours for three times. The autoclaving process was performed three times for 1 hour at 121°C and 2 bar. For heat sterilization, as well as autoclaving a break of 24 hours was done between every sterilization step,

to prevent survival by sporulation. γ-radiation was done at the central group for radiological protection at the University of Giessen, with an estimated dose of 5.06+/-0.2 kGy. Sterilization success of rice straw was determined in slurries as described by (Ho et al., 2011). Therefore, 0.25 g of sterilized straw was mixed with 10 mL of sterile water in 20 mL serum bottles. After preparation the bottles were closed with rubber stoppers, gassed with N_2, and incubated anaerobic at 25°C in dark. The remaining microbial activity after sterilization was followed by measurement of CO_2 in the headspace for 9 days. Furthermore, sterilization success was tested by broad band nutrient enrichment agar (Standard Nutrient-Agar I, Roth). 500 mg of rice straw was placed on agar plates for aerobic incubation, while for anaerobic incubation the straw was completely covered by the agar. The agar plates were incubated either at 30°C, 37°C or room temperature for in darkness 8 days.

Xylan and cellulose are biopolymers of rice straw and mainly converted to CH_4 and CO_2 (Murayama, 1984). Therefore, addition of this two substances was also performed for stimulation of the methanogenic activity. Sufficiency of stimulation by addition of rice straw, as well as xylan and cellulose was also tested in slurries. Therefore, 25 g of the different soil-systems were incubated with 25 mL of sterile water in 120 mL serum bottles, while 25 g of pure Vercelli rice paddy soil served as control. In one line of samples, autoclaved rice straw was added with a concentration of either 0.2, 0.5 or 1 g of straw kg^{-1} of soil, while in another line cellulose and xylan were added with a concentration of 0.1, 0.25 or 0.5 g kg^{-1} each. No stimulants were added for the control. As another control we used each soil-system also without addition of stimulants. Each combination of inoculum and concentration of added stimulant was prepared in triplicates. After preparation, the bottles were closed with rubber stoppers, gassed with N_2 and incubated at 25°C in dark. The potential emission of methane for the different soil-systems was followed by measurement of CH_4 in the headspace every three days, until the emission of methane was stable. Pore water was sampled every week, centrifuged by 12000 rpm for 15 min, and stored at -20°C for further analysis.

2.4 Plant-soil microcosms

30 g of rice seeds (*Oryza sativa*, var. Koral, type japonica, provided by CRA-RIS, Vercelli, Italy; harvested 2009) were treated with 10 mg of the fungicide Aatirm (Stähler Internetional GmbH & Co. KG). Further, the seeds were first sterilized for 1 min with ethanol (75 %) and afterwards for 15 min with sodium hypochlorite solution (2 %). This treatment was repeated for three times. After

every step, the seeds were washed with sterile water. Afterwards, the sterilized seeds were germinated on wet tissue paper for seven days in the green house.

The soil-systems were created by a combination of 20 % (m/m) environmental sample as inoculum and 80 % of inert soil-matrix (m/m, with 79 % sand and 1 % vermiculite). As inocula served, (i) Vercelli rice paddy soil, (ii) digested sludge, (iii) a mixture of digested sludge and Vercelli paddy soil (1:1), and (iv) Philippine paddy rice soil. The synthetic soil-systems were prepared in master mixes and filled in plant pots with a volume of 3 L. For every pot 1185 g of quartz-sand was mixed up with 15 g of vermiculite and 300 g of one of the different inocula. Four pots were prepared for each inoculum, whereas one pot served as backup. For rice paddy soil samples, cellulose and xylan (0.5 g kg_{soil}^{-1}, each) were added and mixed accurate. Arrangements with 100 %Vercelli rice paddy soil served as control. All pots were flooded with water (about 2 cm above soil surface). After seven days of flooding and germination, three rice plants were planted per pot. Directly before planting, a small amount of soil was sampled and stored at -80°C for molecular biological analysis, as well as determination of total soil organic carbon and organic $^{13}C/^{12}C$ ratio. Three weeks after planting, all plants except one were removed from each pot. While the whole experiment, the water level was about 2 cm above soil surface. Pots were placed in the greenhouse with the constant parameters of: lighting 100 kLx at 28°C for 14 hours, 25°C without light for 10 hours per day, and humidity of 80 %. For fertilization with macro- and micro nutrients, 4 mL of Hoagland`s solution (Hoagland and Arnon, 1950) was added to every pot every 14 days, except these pots containing 20 % of digested sludge as inoculum, as well as the control. Pretests have revealed that addition of fertilizer resulted in dying of the rice plants when planted in the digested sludge soil-system. Plant height (of the highest plant segment) was determined every week. Pore water was sampled once per week with Rhizon Flex (10 cm, Rhizosphere research products) directly next to the plant to address the rhizosphere instead of bulk soil. Therefore, the tip of the sampler was placed about 7 cm below soils surface. Sampling volume was about 2 mL, which was collected with evacuated blood collection tubes (3 mL, Terumo Deutschland GmbH). pH-values of the pore water were measured directly after sampling. Afterwards, pore water was stored at -20°C for further analysis.

2.4.1 Pulse-labeling

Pulse-labeling of the plant-soil microcosms with $^{13}CO_2$ was performed at a plant age of 9 weeks, during the reproductive plant stage. For this purpose, a cylindrical labeling chamber (volume 7 L, in-house production) was placed over every plant **(Figure 2.1)**. The chambers were placed in the supernatant pot water to maintain a closed system. A single pulse of 70 mL $^{13}CO_2$ (99 atom %,

Euriso-Top) was injected into the chambers headspace and distributed by a fan, build into the top of the chamber. The consumption of total CO_2 and emission of total CH_4 were analyzed at intervals of 1 hour for 8 hours per day, while their $^{13}C/^{12}C$ ratio was measured twice per day. Concentrations of organic pore water substances, as well as their $^{13}C/^{12}C$ ratio was measured twice per day. Labeling chambers were removed during non-measurement periods.

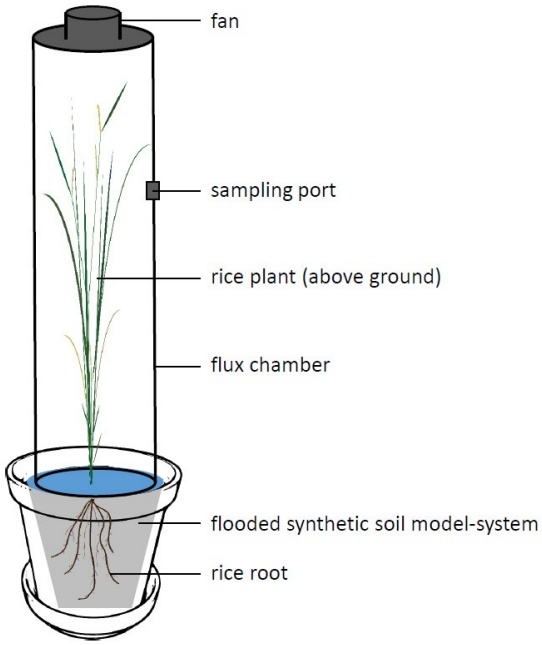

Figure 2.1 | Chamber system for pulse-labeling of rice plant-soil microcosms with $^{13}CO_2$.

The measured $^{13}C/^{12}C$ ratio of CH_4 showed a stable signal after 5 days. Afterwards, all microcosms were sampled destructively, recording weight of above- and blow ground plant biomass. The loose soil of the roots was removed by hand and discarded as bulk soil. The remaining soil particles attached to the roots were removed with a sterile spatula, considered as rhizospheric soil. The rhizospheric soil of one sample was homogenized in sterile aluminum pans (MAGV). Root samples were washed with deionized water three times and sliced into pieces of about 0.5 cm. All samples were stored at -80°C for molecular biological analysis and determination of organic $^{13}C/^{12}C$ ratio. All measurements of total organic carbon and isotopic measurements of $^{13}C/^{12}C$ ratio for soil and plant material were conducted using an elemental analyzer coupled to an isotope-ratio mass

spectrometer (IRMS) at the center for stable isotope research and analysis (KOSI) located at the University of Göttingen.

2.5 Chemical analysis

2.5.1 Measurement of pH-value

The pH-value was determined by use of a microprocessor pH-meter (Type pH539; WTW, Weilheim, Germany), with an InLab Semi-Micro pH electrode (Mettler-Toledo, Giessen, Germany) at room temperature. The pH-meter was calibrated by two point calibration against standard proton-buffer-solutions, with pH-values of 4.01 ± 0.015 and 7.01 ± 0.015. For standardization, the pH-value was converted to the theoretical pH-value at a temperature of 21°C. All classifications of the pH range were done after the standards from the United states Department of Agriculture Natural Resources Conservation Service (Soil Survey Division Staff, 1993).

2.5.2 Analysis of gas samples

The amount of CO_2 and CH_4 in the headspace of the samples was measured via gas chromatograph (GC, GC-8A, Shimadzu, Duisburg, Germany) with a flame ionization detector (FID).

injector:	operating temperature: 160°C
carrier gas:	hydrogen 5.0 (2.0 kg cm^{-2})
column:	HayeSep Q (80/100 mesh, 3 m, 1/8”; Valco Instruments, Houston, United States)
	operating temperature: 120°C
methanizer:	NiCr-Ni-catalysor (20 cm, 1/8”; Chrompack, Middleburg, Netherlands)
	operating temperature: 350°C
detector:	FID

quenching gas:	nitrogen 5.0 (0.3 kg cm^{-2})
combustion gas:	hydrogen 5.0, synthetic air 5.0 (20.5 % oxygen 5.0, 79.5 % nitrogen 5.0; 0.3 kg cm^{-2})

For calibration, a standardized gas mixture of CH_4 = 1000 ppm_v / CO_2 = 1000 ppm_v was used. Injection volume for calibration and measurement was 100 µL. Before sampling from slurries and pore water samples each bottle or column was constantly shaked for three seconds. Analysis was performed using Peak Simple Chromatography Data System (Model 202, SRI Instruments Inc., Las Vegas, United States). The peak area of the chromatogram was used for calculation of analyte concentrations.

$^{13}C/^{12}C$ ratio of CH_4 and CO_2 was determined by gas chromatography coupled to an isotope-ratio mass spectrometer (IRMS), a technique described by (Brand, 1996).

GC:	Isolink Trace GC Ultra (Thermo Fisher Scientific, Karlsruhe, Germany)
injector:	split ratio 1:10
	operating temperature: 150°C
carrier gas:	helium 5.0
	flow rate: 1.5 mL min^{-1}
column:	Pora PLOT Q (27.5 m length, 0.32 mm diameter, film 10 µm, Varian, Palo Alto, CA, United States)
	operating temperature: 30°C
GC/C interface:	GC Isolink 1030 (Thermo Fisher Scientific)
	oxidation-reactor temperature: 940°C
detector:	IRMS: Delta V Advantage (Thermo Fisher Scientific)

As reference gas served CO_2 (isotope purity 99,998%; Air Liquide), calibrated with working standard methyl stearate (Merck). Analysis of the chromatogram occurred with the software Isodat (Version 3.0, Thermo Fisher Scientific).

2.5.3 Analysis of organic pore water substances

Organic pore water substances, which were not degassed to the head space, have been analyzed by high performance liquid chromatography (HPLC).

HPLC system:	pump: S 1125 HPLC Pump System (Sykam GmbH, Eresing, Germany)
	oven: Mistral (Spark, Emmen, Netherlands)
autosampler:	S 5200 Sample Injector (Schambeck SFD GmbH, Bad Honnef, Germany)
column:	Animex HPX-87H (Biorad, Hercules, California, United States)
	operating temperature: 35 C
eluent:	sulfuric acid (1 mM)
	flow rate: 0.5 mL min^{-1}
detector:	UV: UV detector 2500 (Sykam)
	RI: ERC-7512 (Erma optical works, Tokyo, Japan)

An injection volume of 100 µL was used. Resulting chromatograms were analyzed with the software clarity (Version 4.0.3.843, Techlab GmbH, Braunschweig, Germany). The analyte concentrations were determined by using of five point calibration, with standard concentrations between 2 mM and 10 mM. The equimolar standards contained the substances: acetate, butyrate, formate, glucose, heptanoic acid, hexanoic acid, isobutyrate, isocaproic acid, isovalerate, lactate, propionate, pyruvate, and valerate.

For determination of the $^{13}C/^{12}C$ ratio for organic substances we used HPLC linked to an IRMS by an LC-interface (HPLC-IRMS), a technique described by (Krummen et al., 2004).

HPLC system:	pump: Finnigan™ Surveyor MS Pump (Thermo Fisher Scientific)
	oven: Mistral (Spark)
interface:	Finnigan™ LC IsoLink (Thermo Fisher Scientific)
	operating temperature: 99°C
IRMS:	Finnigan™ Delta V Advantage (Thermo Fisher Scientific)
autosampler:	Finnigan™ Surveyor Autosampler plus (Thermo Fisher Scientific)
column:	Nucleogel Sugar 810 H (Macherey-Nagel)
	operating temperature: 35°C
eluent:	sulfuric acid (5 mM)
	flow rate: 0.3 mL min^{-1}
oxidant:	$Na_2S_2O_4$ solution (50 g L^{-1})
	phosphoric acid (1.5 M)
	flow rate: 50 μL min^{-1}, each

A sampling volume of 100 μL was used. Analysis of the chromatogram was performed with the software Isodat (Version 3.0, Thermo Fisher Scientific). The equimolar standards contained the substances: acetate, butyrate, formate, glucose, heptanoic acid, hexanoic acid, isobutyrate, isocaproic acid, isovalerate, lactate, propionate, pyruvate, and valerate. As reference for $^{13}C/^{12}C$ ratio served CO_2 (isotope purity 99,998%; Air Liquide), calibrated with working standard methyl stearate (Merck).

2.5.4 Calculation of gas concentrations, emission rates and carbon isotope ratios

The gas concentration c was measured in parts per million by volume (ppm_V) and converted to amount of substance n using the ideal gas law (1).

$$n\,[nmol] = \frac{c[ppm_v] \cdot p_0[bar] \cdot V[L] \cdot 10^3}{R[bar\ L\ K^{-1}\ mol^{-1}] \cdot T[K]} \qquad (1)$$

n: amount of substances

c: gas concentration

p_0: standard atmospheric pressure (1.01325 bar)

R: universal gas constant ($8.3145 \cdot 10^{-2}$ bar L K^{-1} mol^{-1})

T: temperature (298.15 K)

Beside concentrations, the analysis by GC-IRMS and HPLC-IRMS also revealed the isotopic ratio R for carbon compounds (2).

$$R_C[\%] = \frac{^{13}C}{^{12}C} \cdot 100 \qquad (2)$$

^{13}C: number of ^{13}C isotopes of an analyte

^{12}C: number of ^{12}C isotopes of an analyte

From this output data, the relative amount of ^{13}C isotopes F for carbon compounds was calculated (3).

$$F_{^{13}C}[atom\ \%] = \frac{^{13}C}{^{12}C + ^{13}C} \cdot 100 = \frac{R_C}{R_C + 1} \cdot 100 \qquad (3)$$

To determine the decrease of $^{13}CO_2$ in the headspace of the labeling chambers, the amount of substance for $^{13}CO_2$ was calculated for different time points (4).

$$n_{^{13}CO_2}\,[\mathrm{nmol}] = \frac{F_{CO_2}\,[\mathrm{atom\ \%}] \cdot n\,[\mathrm{nmol}]}{100} \qquad (4)$$

To determine ^{13}C-labeling of carbon compounds, the ^{13}C [atom % excess] value was calculated (5), which is defined as the relative amount of isotopes about a base level. As a base level we used the isotopic ratio of the analyte without pulse-labeling.

$$^{13}C\,[\mathrm{atom\ \%\ excess}] = F_{^{13}C}(\mathrm{labeled}) - F_{^{13}C}(\mathrm{base}) = \left[\left(\frac{R_C}{R_C+1}\right)_{\mathrm{labeled}} - \left(\frac{R_C}{R_C+1}\right)_{\mathrm{base}}\right] \cdot 100 \qquad (5)$$

The amount of substance for an analyte, which was formed by degradation of ^{13}C-labeled carbon compounds, was calculated by use of the ^{13}C atom % excess value (6).

$$n_{^{13}C}\,[\mathrm{nmol}] = \frac{^{13}C\,[\mathrm{atom\ \%\ excess}] \cdot n\,[\mathrm{nmol}]}{100} \qquad (6)$$

The emission rate of total methane was calculated in relation to dry weight of the soil (7), whereas emission rate of methane formed by root derived carbon was calculated in relation to the dry root biomass (8). Mass of the soil was assumed as total mass of the soil-system, means sum of inoculum and inter matrix.

$$\text{total } CH_4 \left[\text{nmol } g_{dw,soil}^{-1} \text{ } h^{-1}\right] = \frac{\Delta n_{CH_4} \text{ [nmol]}}{m\left[g_{dw,soil}\right] \cdot \Delta t \text{ [h]}} \quad (7)$$

$$^{13}CH_4 \left[\text{nmol } g_{dw,root}^{-1} \text{ } h^{-1}\right] = \frac{\Delta n_{^{13}CH_4} \text{ [nmol]}}{m\left[g_{dw,root}\right] \cdot \Delta t \text{ [h]}} \quad (8)$$

Δn: difference in amount of substances between two time points of measurement

Δt: time interval between two time points of measurement

The contribution of recently plant-assimilated carbon to methane emission was calculated by use of the amount of substances (9).

$$R_m\left[\%\right] = \frac{n_{^{13}CH_4} \text{ [nmol]}}{n_{^{12+13}CH_4} \text{ [nmol]}} \cdot 100 \quad (9)$$

Unless otherwise noted, plots were created by the mean and standard deviation values of the triplicates.

2.6 Molecular biological analysis

2.6.1 DNA extraction

Root samples were crushed with a mortar under presence of liquid nitrogen. 0.25 g of rhizospheric soil and root samples were used for DNA extraction with the NucleoSpin®Soil Kit (Macherey-Nagel, Düren, Germany), using the instructions recommended by the manufacturer with some minor modifications. To increase the amount of extracted DNA, the lysis of microbial cells was performed twice. Since the extraction kit provides two buffers for lysis, both were tested and extraction of all samples was performed with buffer SL2 and addition of 150 µL lysis enhancer (both provided with the kit).

2.6.2 Real-time quantitative PCR (qPCR)

qPCR was used to quantify the gene copy numbers of the phylogenetic and functional marker genes. Following primer combinations were used, addressing the different target genes: Ba519f/Ba907r (Lane, 1991) for bacterial 16S rRNA genes, Ar364f/Ar934br (Burggraf et al., 1997; Großkopf et al., 1998a) for archaeal 16S rRNA genes, mlas-mod/mcrA-rev (Steinberg and Regan, 2008; Angel et al., 2011) for *mcrA* genes, and A189f/mb661 (Holmes et al., 1995; Costello and Lidstrom, 1999) for *pmoA* genes. All qPCR reactions were prepared on ice with minimal exposure to light. Before analysis by qPCR, all DNA samples were diluted (1:200). The measurement was performed in triplicates. Standards, containing the known numbers of gene copies for the target gene, were diluted serially and used for calibration in each reaction. The standards were amplified from *Eschericha coli* strain K12 for 16S rRNA genes, from *Methanosarcina barkeri* for archaeal 16S rRNA and *mcrA* genes, and from *Methylomonas* sp. for *pmoA* genes, in dilution series from 10^1 up to 10^7 gene copies.

qPCR reactions were performed using an CFX Connect™ Real-Time PCR Detection System (Bio-Rad, München, Germany), and contained the following components: 12.5 μL Sybr® Green Ready Mix (Sigma-Aldrich, Karlsruhe, Germany), 0.125 μL of each primer (100 μM), 4 μL $MgCl_2$ (25 mM, Sigma-Aldrich), 3.25 μL PCR-water (Sigma-Aldrich), and 5 μL of diluted template DNA, with a total volume 25 μL for bacterial 16S rRNA genes; 12.5 μL Sybr® Green Ready Mix, 0.5 μL of each primer (33 μM), 3 μL $MgCl_2$ (25 mM), 3.5 μL PCR-water, 0.1 μL BSA (20 μg μL^{-1}, Roche), and 5 μL of diluted template DNA, with a total volume 25 μL for archaeal 16S rRNA genes; 12.5 μL Sybr® Green Ready Mix, 0.25 μL of each primer (25 μM), 3.5 μL $MgCl_2$ (25 mM), 3.5 μL PCR-water, 0.1 μL BSA (20 μg μL^{-1}), and 5 μL of diluted template DNA, with a total volume 25 μL for *mcrA* genes; 7.5 μL KAPA SYBR® FAST Master Mix (Kapa Biosystems, Boston, United States), 0.3 μL of each primer (20 μM), 1.9 μL PCR-water, and 5 μL of diluted template DNA, with a total volume 15 μL for *pmoA* genes. The following qPCR programs were used: 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 for bacterial 16S rRNA genes; 94°C for 6 min, followed by 40 cycles of 94°C for 35 s, 66°C for 30 s, 72°C for 45 s and 86.5°C 10 s for archaeal 16S rRNA genes; 94°C for 5 min, followed by 40 cycles of 95°C for 30 s, 57°C for 45 s, 72°C for 30 s, and 84°C for 10 s, for *mcrA* genes; 95°C for 2 min, followed by 39 cycles of 95°C for 10 s, 60°C for 20 s, and 84°C for 7 s, for *pmoA* genes. Melting curves were measured from 75-95°C for 16S rRNA and *mcrA* gene samples, and 65°C-95°C for *pmoA* samples. Resulting data was analyzed using CFX Manager Software (Version 3.0).

2.6.3 PCR amplification for illumina sequencing

Identification of the microbial rhizospheric community was done via Illumina© MiSeq amplicon Sequencing, with pair-end reading of 300 nt. To combine different target genes in one sequencing run, a universal barcode method was developed, based on (Boutin-Ganache et al., 2001), to tag amplicons of the same samples with unique barcodes. Therefore, the forward and reverse primers were tailed with a M13-sequence (5'-CAC GAC GTT GTA AAA CGA C-3') on the 5' end. Combinations of the used M13-tagged primers are shown in **Table 2.1**. Target genes were amplified using the following reactions, with a total volume of 50 μL: 1 μL AccuPrime™ DNA polymerase (Life Technologies, Darmstadt, Germany), 5 μL AccuPrime™ PCR Buffer II, 1 μL of each primer (10 μM), 40 μL PCR-water, and 2 μL of template DNA for bacterial 16S rRNA genes; 1 μL AccuPrime™ DNA polymerase, 5 μL AccuPrime™ PCR Buffer II, 1 μL of each primer (10 μM), 0.5 μL BSA (20 μg μL^{-1}, Roche), 39.5 μL PCR-water, and 2 μL of template DNA for *mcrA* genes. The PCR conditions were as following: initial denaturation (3 min at 95°C), 5 cycles (30 s at 95°C, 45 s at 48°C with a ramp of 0.1°C/s to 68°C for 35 s), followed by another 30 cycles (30 s at 95°C, 45 s at 55°C, 35 s at 68°C), and terminal extension (10 min at 68°C) for *mcrA* genes; initial denaturation (3 min at 95°C), 30 cycles (30 s at 95°C, 20 s at 48°C, 35 s at 68°C), and terminal extension (10 min at 68°C) for 16S rRNA genes. Two separate 50 μL reactions were used per sample, pooled afterwards, and purified using the GenElute™ Gel Extraction Kit (Sigma-Aldrich), with instructions recommended by the manufacturer. Unique barcodes of 8 nt length **(Table S2.1)** were also tagged with a M13-sequence (on the 3' end) and attached to the gene amplicons, using a second PCR. The barcode-PCR reactions contained the following substances for all genes: 1 μL AccuPrime™ DNA polymerase, 5 μL AccuPrime™ PCR Buffer II, 2 μL of M13-barcode primer (10 μM), 0.5 μL BSA (20 μg μL^{-1}, Roche), 40.5 μL PCR water, and 1 μL of amplicon DNA. The PCR conditions were as followed: initial denaturation (3 min at 95°C), 8 cycles (30 s at 95°C, 30 s at 48°C, 35 s at 68°C), and terminal extension (10 min at 68°C). The barcoded amplicons were purified using the GenElute™ PCR cleanup Kit (Sigma-Aldrich) with instructions recommended by the manufacturer.

To test the sufficiency of the universal barcode system, M13-*mcrA* amplicons were created, combined with an M13-barcode, ligated into an pGEM®-T-easy vector system (Promega, Mannheim, Germany) and afterwards transformed into competent *Escherichia coli* cells (JM109, Promega). 10 randomly selected clones were sequenced on a ABI 373 DNA sequencer (Applera, Darmstadt, Germany) using the BigDye® Terminator v3.1 Cycle sequencing Kit (Life Technologies), with instructions recommended by the manufacturer. All tested clones contained

primer binding sites, M13- and barcode sequences on both sides while the M13- and barcode sequence was in a reverse and complimentary orientation on one end. Since the barcoding was sufficient, the DNA amount of all prepared and barcoded amplicons was determined by the use of a Qubit Flourometry (Thermo Fisher Scientific, Bremen, Germany). Afterwards, equimolar amounts of the amplicons were mixed and sequenced at the Beijing Genomic Institute Tech Solutions Center (Hong Kong) using the MiSeq 300 nt paired end Illumina sequencing technology.

Table 2.1 | Barcoded oligonucleotide primers used for illumina amplicon sequencing.

target gene	primer name	primer sequence (5' to 3')	reference for untagged primer
mcrA	M13-mlas	CAC GAC GTT GTA AAA CGA CGG TGG TGT MGG DTT CAC MCA RTA	Steinberg and Regan, 2008
	M13-mcrA-rev	CAC GAC GTT GTA AAA CGA CCG TTC ATB GCG TAG TTV GGR TAG T	
bacterial 16S rRNA gene	M13-343Fmod	CAC GAC GTT GTA AAA CGA CTA CGG GWG GCW GCA	Köhler et al., 2012
	M13-784Rmod	CAC GAC GTT GTA AAA CGA CGG GTM TCT AAT CCB KTT	

* nucleotide coding. A = Adenine, C = Cytosine, G = Guanine, T = Thymine; wobble nucleotides: M = A/C, R = A/G, K = G/T, V = A/C/G, D = A/G/T, B = C/G/T, N = A/C/G/T.

2.7 Data manipulation and analysis

Paired end illumina sequencing was performed in repeated runs, producing various forward and reverse data sets containing sequencing- and quality data in FASTQ-format files. The raw illumina sequencing data was tested for read quality by use of the FastQC software (Version 0.11.4, Babraham Bioinformatics). All separated forward sequences were combined using the software mothur (Version 1.36.0, (Schloss et al., 2009)), as well as all reverse sequences. Since use of different calculation methods would create different quality-values, all data preparation up to OTU clustering was performed only using the Usearch- (Edgar, 2010) and Uparse algorithms (Edgar, 2013), implemented in the Usearch software package (Version 8.1.1861, drive5 – Bioinformatics software and services). The sequence analysis was performed based on the OTU analysis pipeline (including the provided python scripts) provided by the software producer (http://drive5.com/usearch/manual/uparse_pipeline.htm, as of May, 2016). Therefore, forward- and

reverse sequences were merged using the overlapping paired ends. Sequences of the different gene amplicons in the respective samples were separated by use of the forward primer-and barcode sequences. The primer- and barcode sequences were trimmed from the amplicon sequences in the same process step. This step was performed in origin orientation of the sequence, as well as in the complementary-reverse orientation, since illumina reads are not directed. Afterwards, sequences were filtered for quality and globally trimmed. Therefore, sequences with an expected error per base E larger than 1 were removed, as well as sequences which did not fulfill the length criteria of the amplicons.

After the step of dereplication, analysis of the sequences was performed in different ways with respect to their gene type. Furthermore, two data sets were created for every sample, while for one of them the singletons were removed. Unique 16S bacteria rRNA gene sequences were clustered to OTUs with a similarity of 97 %. Chimeras were removed and remaining reads were mapped to OTUs with 97 % of similarity cutoff. Their taxonomy was assigned by use of the software QIIME (Version 1.9.0, (Caporaso et al., 2010)) and the Greengenes 16S rRNA gene database (http://greengenes.secondgenome.com, from May, 2013), as reference.

mcrA gene sequences were also clustered to OTUs by similarity of 97 %, but the created OTUs were pooled afterwards by a similarity of 84 %, according to (Yang et al., 2014). Chimera filtering occurred against a *mcrA* database created by (Angel et al., 2011). The reads were mapped to OTUs by similarity of 84 %. The taxonomy was assigned using the ARB software package (Version 6.0.2, (Westram et al., 2011)). Therefore, a *mcrA* gene database was created on the basis of a protein based tree, calculated from pure culture *mcrA* sequences generated by (Angel et al., 2011). Further, 382 new *mcrA* gene sequences, originated form National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/nuccore), were translated to protein sequences using the Framebot online tool (Wang et al., 2013). These new sequences were aligned using the ClustalW method (Thompson et al., 1994) and added to the basis tree by parsimony (Ludwig et al., 2004). Alignment of the imported sequences was performed manually against their closest relatives of the base tree. Afterwards, all sequences were used to calculate a new tree with RaxML 7.04 using rapid hill climbing algorithm and PROTMIX-JTT evolutionary model (Stamatakis, 2006). This new tree was used as reference for taxonomic assignment of *mcrA* amplicon illumina reads which were assigned in the same way as described above.

2.8 Statistical analysis

Statistical analysis was done using the software environment R (Version 3.2.4, R Development Core Team, 2011). All levels of significance were defined at $p \leq 0.05$. Data sets of chemical analysis and quantitative PCR were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965; Royston, 1982). Further, a one-way analysis of variance (ANOVA) was calculated and means of the different samples for the levels of different measured parameters were compared by post hoc Tukey test (Miller Jr., 1981), using the R-package *stats*. Unless otherwise noted, plots were created by the mean and standard deviation values of the triplicates.

The processed OTU data sets for bacterial 16S rRNA and *mcrA* genes from illumina sequencing were used for calculation of diversity, and statistical analysis. The species richness (Chao, 1984) in samples was estimated with the R-package *phyloseq* (Version 1.4-5, (McMurdie and Holmes, 2013)), sample coverage (Good, 1953) with the R-package *entropart* (Version 1.4-6), diversity index (Chao and Shen, 2003) with the R-package iNEXT (Version 2.0.12), and species evenness (Legendre and Legendre, 1998) with *asbio* (Version 3.40), by use of the data set also containing the singletons. The relative abundance was calculated from all sequences within a tested gene on different taxonomic levels without singletons, using the R-package *phyloseq*. Ordination in form of canonical correspondence analysis (CCA, (McGarigal et al., 2013)) was performed on OTU-level by use of the *vegan* package (Version 2.2-1, (Oksanen et al., 2013)). Terms of environmental parameters were tested by ANOVA, and further by ordistep function (Oksanen et al., 2013). Identification of indicator species (Dufrene and Legendre, 1997) was performed by use of the package *indiscpecies* (Version 1.7.4). The relative abundance of indicator species according to important environmental parameters were plotted in a heatmap by use of the R-package *gplots* (Version 3.0.1) and also used for a cluster analysis (Murtagh, 1985) based on the Bray-Curtis distance (Bray and Curtis, 1957). The ternary plots were created within the R-package *vcd* (Version 1.3-2).

III. Results

The aim of this study was to investigate the link between microbial colonization of the rice rhizosphere and its influence on the emission of methane by degradation of root derived carbon. To understand the process of root colonization it was necessary to provide different initial microbial communities of different environmental samples as seed bank for rice root colonization. Therefore it was essential to establish a system which (i) minimized the abiotic factors of the different environmental samples, but still provided different initial microbial communities; (ii) contained a healthy rice plant, placing their roots in direct proximity to the microbial seed bank; and (iii) allowed to follow the translocation of carbon from photosynthetic CO_2-fixation by the plant through the release of rhizodeposits to microbial degradation to methane. Such rice planted soil-microcosms were previously developed by (Pump and Conrad, 2014; Pump et al., 2015). Based on this approach we also used inoculated synthetic soil-systems to provide different initial microbial communities for colonization of the rice rhizosphere, while coincidentally minimizing the abiotic factors of the different inocula.

However, it was necessary to modify these sand-vermiculite amended microcosms to establish reliable plant-soil model-systems, which could be used with a variety of inocula beside rice paddy soil. For this purpose we created modified synthetic soil model-systems and tested them in different ways for stimulation of the methanogenic activity.

3.1 Synthetic soil model-systems

Different environmental samples were used as origin for different initial microbial communities, ready for colonization of rice roots. Since not only the initial microbial communities were different between the environmental samples, it was necessary to minimize the differences in their physical and chemicals parameters. Therefore, synthetic microbial communities were created in artificial soil-systems by combination of 20 % environmental sample as inoculum and 80 % inert soil-matrix. Analogous to a natural soil type, this combination of a certain inoculum and inert matrix is considered as distinct soil-system. However, since the amount of original environmental sample in the synthetic soil-systems was reduced from 100 % to 20 %, the resulting loss in microbial activity was also thought to reduce the potential emission of methane. To compensate for this, the methanogenic activity was stimulated by addition of rice straw or xylan and cellulose.

3.1.1 Efficiency of rice straw sterilization

Since it was necessary to obtain the natural composition of the microbial communities of the different inocula, rice straw was sterilized with different methods. To determine the sterilization success, the remaining microbial degradation activity on the rice straw was measured by quantification of the CO_2 formation **(Figure 3.1)**. The remaining microbial activity was highest for straw treated by γ-radiation. Although, microbial formation of CO_2 was reduced in comparison to non-sterilized rice straw (about $8 \cdot 10^6$ ppm_v), γ-radiation showed an insufficient sterilization effect. Exposure to heat of 140°C reduced the microbial activity more effectively than radiation, dependent on the duration of the heat treatment. The difference between 12 h and 24 h of heat exposure was not as high as between 6 h and 12 h. The remaining microbial activity of rice straw, treated by autoclaving, was quite lower compared to the other tested sterilization methods. The autoclaving process was by far the most effective method for sterilizing rice straw, with CO_2 values similar to the negative control which contained only sterile water.

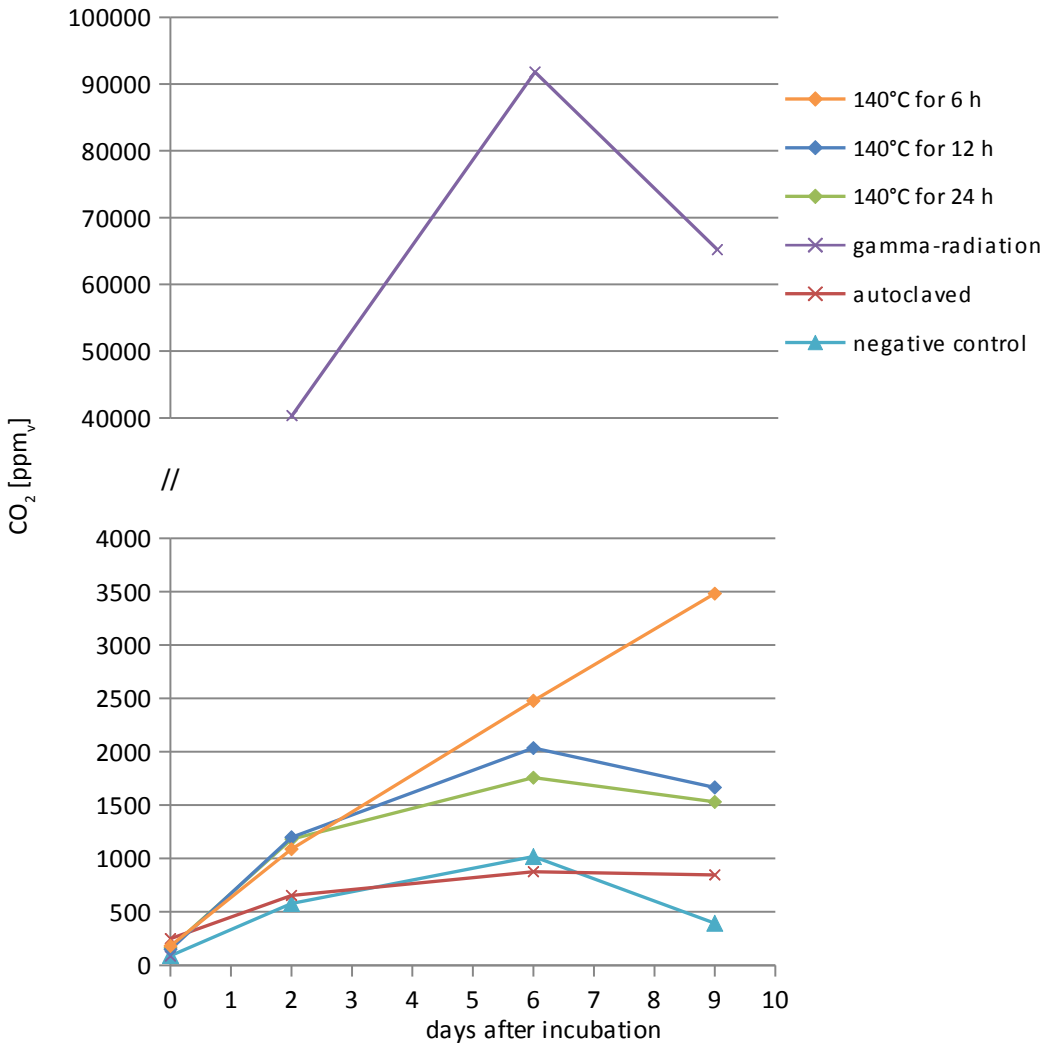

Figure 3.1 | CO_2 measurement for determination of remaining microbial activity from anaerobic incubated rice straw slurries, after different sterilization processes.

Verification of residual microorganisms on treated rice straw was further determined by growth on broad band nutrient agar **(Table 3.1)**. Except for autoclaved rice straw, all other samples were still colonized by microorganisms after sterilization, noticeable by a distinct growth on nutrient agar under anaerobic conditions after 9 days of incubation. After treatment with γ-radiation the rice straw still possessed residual microorganisms, able to grow after 1 day of incubation under both anaerobic and aerobic conditions. In summary, sterilization of rice straw by autoclaving was the only method which adequately reduced microbial activity, as well as the maintaining microbial

growth potential. Therefore, only rice straw treated by the autoclaving process was used for further experiments.

Table 3.1 | Number of microbial colonies observed during incubation of rice straw, sterilized with different methods, on nutrient agar to determine sterilization success. No microbial growth is indicated by n.g.

		aerobic incubation				anaerobic incubation			
		days after incubation				days after incubation			
straw treatment	incubation temperature	1	2	6	9	1	2	6	9
140°C for 6h	RT	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	20 - 50
140°C for 6h	30°C	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
140°C for 6h	37°C	n.g.	n.g.	n.g.	n.g.	n.g.	1	1	20 - 50
140°C for 12h	RT	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	5	5
140°C for 12h	30°C	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	6	10 - 20
140°C for 12h	37°C	n.g.	n.g.	n.g.	n.g.	n.g.	2	20 - 50	20 - 50
140°C for 24h	RT	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
140°C for 24h	30°C	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
140°C for 24h	37°C	n.g.	n.g.	n.g.	n.g.	1	1	1	20 - 50
γ-radiated	RT	n.g.	8	10	> 100	-	1	20 - 50	> 100
γ-radiated	30°C	10	20 - 50	20 - 50	> 100	10	> 100	> 100	> 100
γ-radiated	37°C	3	7	10	20 - 50	10	> 100	> 100	> 100
autoclaved	RT	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
autoclaved	30°C	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
autoclaved	37°C	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	1

3.1.2 Potential methane emission

Methanogenic activity of different soil-systems was stimulated by the addition of either autoclaved rice straw or xylan and cellulose. The potential methane emission of the different soil-systems was measured in slurries **(Figure 3.2)**. When incubated under anaerobic conditions all systems showed production of methane. The emission of methane from both rice paddy soils was stimulated by addition of xylan and cellulose. For Vercelli rice soil, any addition of rice straw resulted in a higher methane emission rate, whereas only addition of rice straw with 1.0 g kg^{-1} stimulated methane emission in the Philippine soil. Methane formation by addition of 1.0 g kg^{-1} xylan and cellulose was higher than any other approach in both of the rice paddy soils, with methane emission rates equal

to 100 % soil controls. Therefore, addition of xylan and cellulose in total was shown to be more effective for rice paddy soils compared with addition of rice straw. The potential methane emission in total was nearly similar between the two different rice paddy soils.

In contrast, there was no stimulation of the methane emission by addition of stimulants to digested sludge and mixed inocula samples. Formation of methane from synthetic soil-systems based on these two inocula were similar to each other and far higher compared to the rice paddy soils. While for both rice paddy soils and digested sludge samples potential methane emission from synthetic soil-systems was about a fifth compared with the 100 % control, methane formation from synthetic soil-systems with mixed inoculum was in a range of a half compared to the control.

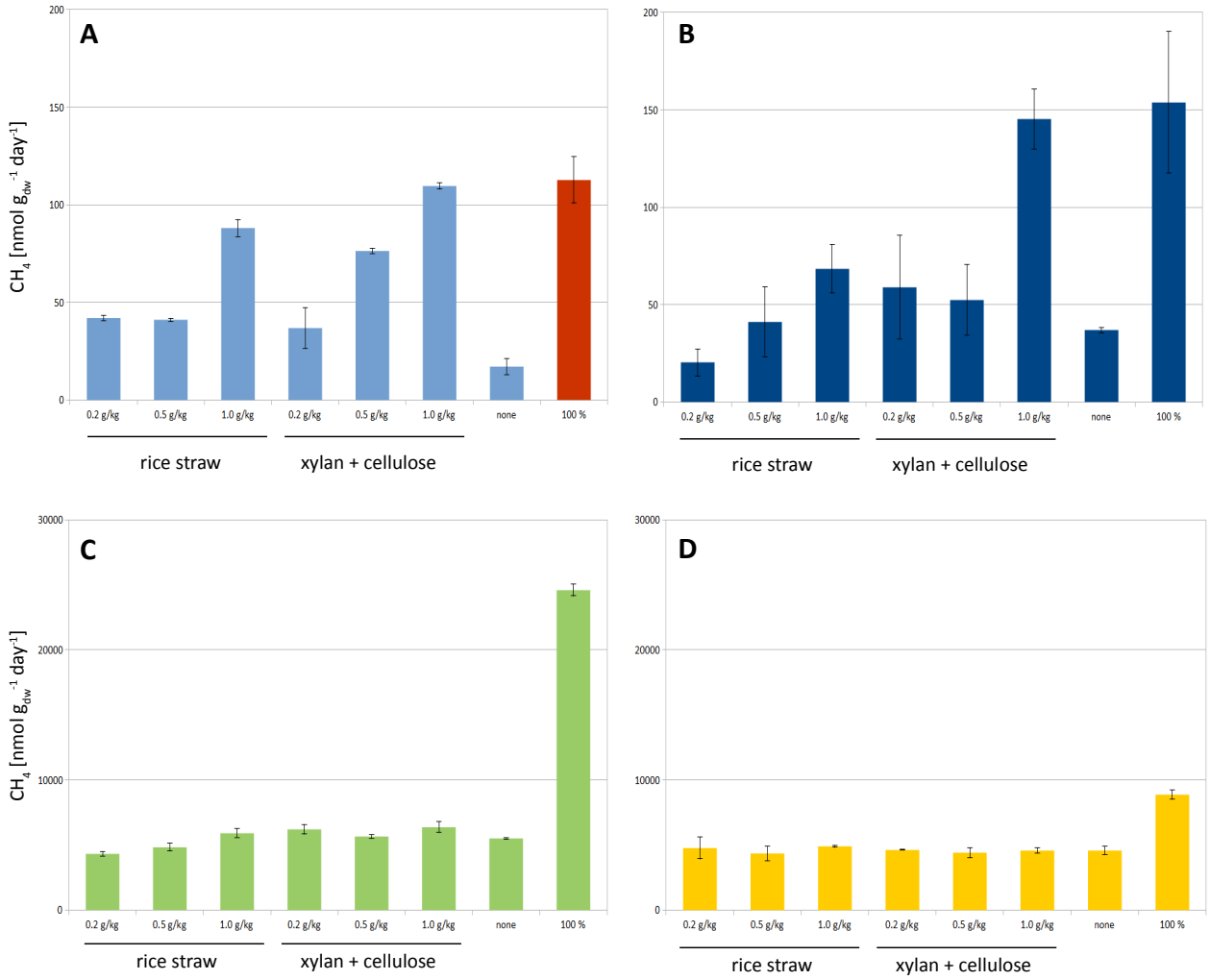

Figure 3.2 | Potential methane emission rates of different inocula (**A**: Vercelli rice paddy soil; **B**: Philippine rice paddy soil; **C**: digested sludge, **D**: mixture of digested sludge and Vercelli rice paddy soil, 1:1) used in synthetic soil model-systems. Different amounts of rice straw or xylan and cellulose (1:1) were added for stimulation in contrast to 100 % of inoculum as control.

3.1.3 Characteristics of synthetic soil-systems

Since stimulation of potential methane emission was highest with 1.0 g kg^{-1} xylan and cellulose, synthetic soil-systems based on rice paddy soil were treated in this way. The remaining samples, as well as the 100 % Vercelli rice paddy soil control were not treated with stimulants. Carbon and nitrogen content, as well as pH value of the different synthetic soil-systems, are shown in **Table 3.2**. Values of carbon and nitrogen were almost similar for Vercelli and Philippine rice soil,

while values of digested sludge and the mixed inoculum systems were higher than the control. The pH value of Philippine soil and digested sludge systems were slightly higher than these of Vercelli soil, mixed inoculum and the control, but all pH values were within the range of a neutral soil pH.

Table 3.2 | Characteristics of the synthetic soil-systems.

	20 % Vercelli rice soil*	20 % Philippine rice soil*	20 % digested sludge	20 % mixed	100 % Vercelli rice soil
C [%]	0.41 ± 0.00	0.50 ± 0.15	5.39 ± 0.46	3.08 ± 0.16	1.22 ± 0.00
N [%]	0.02 ± 0.00	0.02 ± 0.00	0.89 ± 0.09	0.46 ± 0.04	0.11 ± 0.01
pH value	6.85 ± 0.08	7.10 ± 0.10	7.07 ± 0.23	6.79 ± 0.22	6.74 ± 0.01

* values were determined after addition of xylan and cellulose.

3.2 Rice-planted synthetic soil microcosms

Rice-planted microcosms were created by planting rice plants in a certain synthetic soil-system. Although methane emission occurred in all approaches of the slurry experiment, formation of methane from plant-soil microcosms based on Philippine rice soil was only slightly above detection limit and at least one order of magnitude below the other samples (data not shown). Since methane emission is a crucial link between root colonization and formation of methane from root derived carbon, Philippine soil samples were not further analyzed. Vercelli soil samples will henceforth be referred to as rice paddy soil for short. Rice-soil microcosms were created by use of synthetic soil-systems with rice paddy soil, digested sludge and a 1:1 mixture of both as inocula. Microcosms with 100 % Vercelli rice paddy soil served as a control.

Plant height was slightly affected by the type of synthetic soil-system **(Figure 3.3)**. After a short lag phase, rice planted in synthetic soil with mixed inoculum showed almost the same growth as the 100 % control, while soil-systems based on rice paddy soil showed a slight lag in growth rates starting at the late vegetative stage. The systems based on digested sludge showed a distinct delay in growth until the late vegetative stage but caught up on that delay in subsequent growth stages. While rice plants in mixed inocula systems reached the same total plant height as the

control during the reproductive stage, rice plants from rice paddy soil reached about 80 % of total size, and plants from digested sludge systems about 70 % compared to the control.

Above ground plant biomass of all plants grown in synthetic soil-systems was in approximately the same range, but below the biomass of the control. Rice plants planted in soil-systems with mixed inoculum were the only ones which showed some variability in the above ground biomass. The below ground plant biomass of all soil-systems was also lower than the control, while that of the systems with mixed inoculum was higher compared with the other soil-systems. Nevertheless, appearance of all plants was healthful and there were no signs of nutrient deficiency or stunted development.

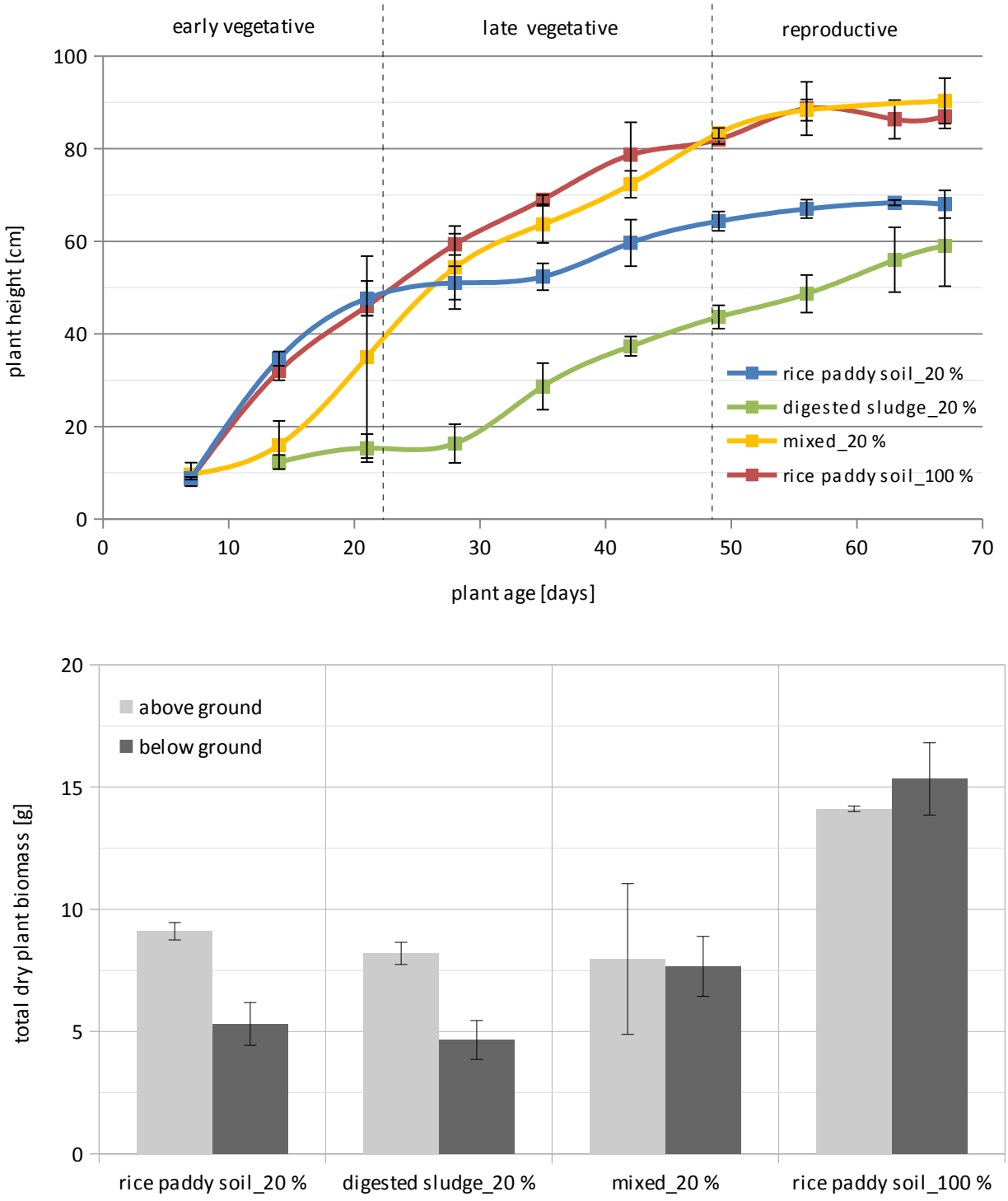

Figure 3.3 | Growth of rice plants in plant soil model-systems with different inocula. **Above:** Plant height during cultivation in the green house, dashed lines indicating different growth stages of the rice plants. **Below:** Dry plant biomass at the end of the reproductive growth stage.

Directly after flooding of the soil-systems, the pore water pH values of all different inocula were within the range of a neutral pH for soil **(Figure 3.4)**. None of the samples showed a direct response in pH value to the planting of the rice plants after 7 days. The pH value of rice paddy soil-systems and the control were relatively stable across the whole experiment and always within a range of a neutral soil pH value. The pH value of microcosms with mixed inoculum reached a neutral soil pH after 30 days, temporarily increasing to a range of slightly alkaline in the preceding period. The pore water pH value of microcosms based on digested sludge increased after flooding from neutral up to a value of slightly alkaline until the end of the experiment.

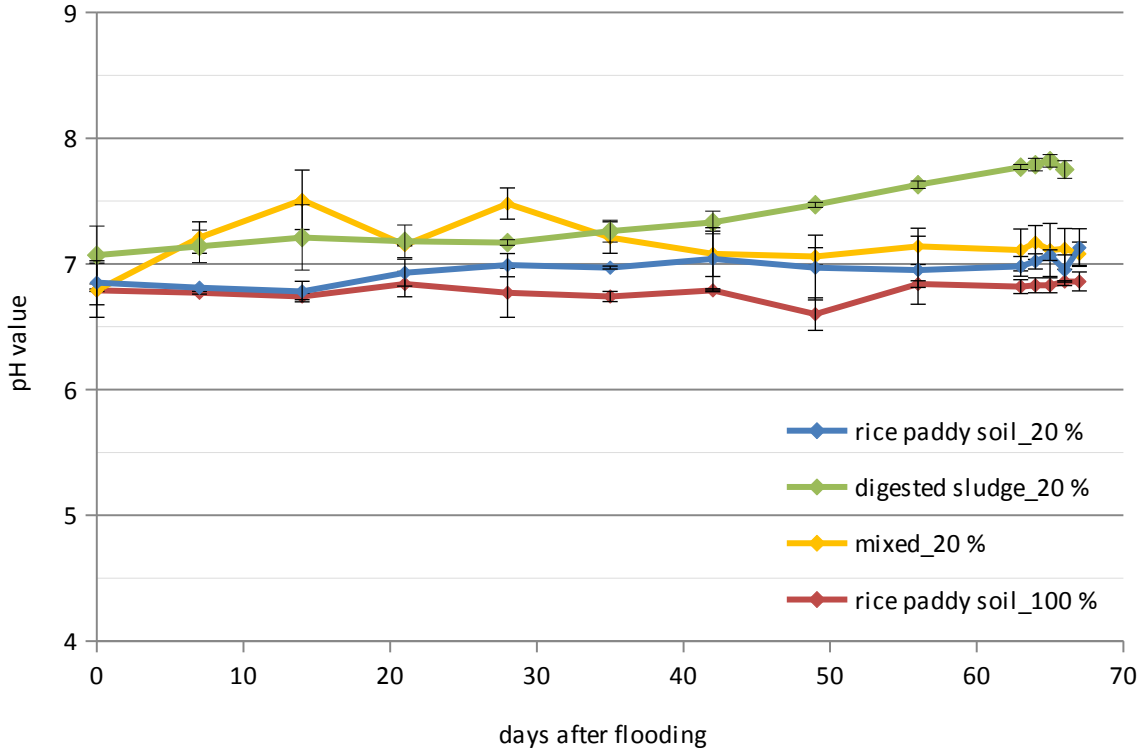

Figure 3.4 | pH values of pore water in plant-soil microcosms with different soil-systems.

Concentrations of organic acids in pore water were stable about the late vegetative and reproductive plant stage in all microcosms. Low concentrations of acetate could be detected in pore water of all soil-systems **(Figure 3.5)**. Acetate concentrations in rice paddy soil and mixed inoculum samples were slightly higher compared to the digested sludge samples. Acetate

concentrations in all synthetic soil-systems were overall higher compared with the 100 % soil control.

Concentrations of propionate in microcosms based on digested sludge were far higher compared to the other soil-systems, as well as the control. Formate could only be detected in the digested sludge system (0.52 ± 0.13 mM), while pyruvate was only present in very low amounts (7.93 ± 0.01 μM) in microcosms containing synthetic rice paddy soil. Further potential organic pore water substances were below the detection limit.

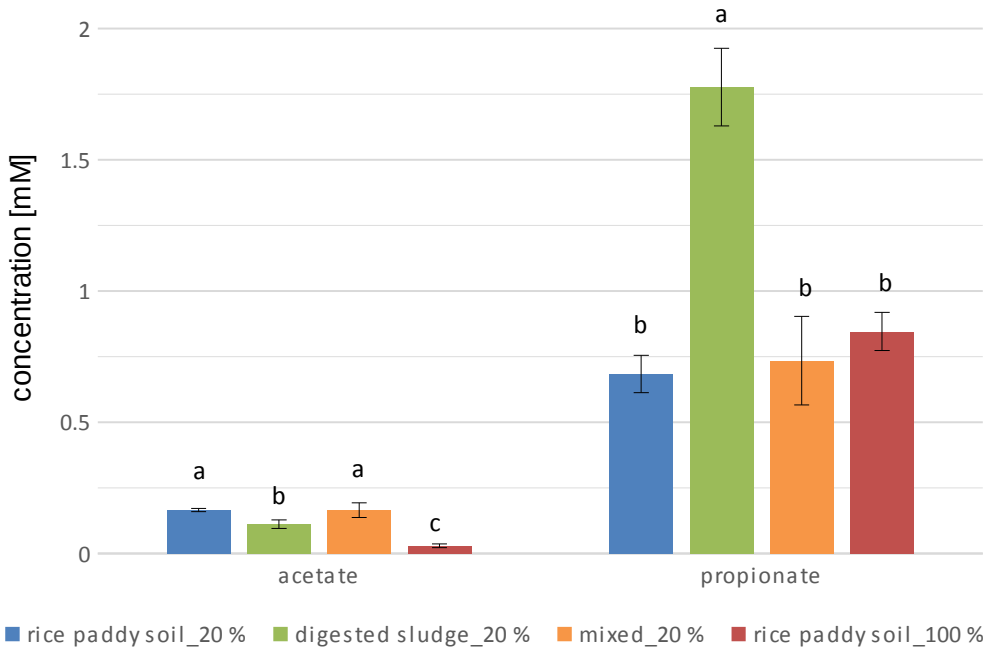

Figure 3.5 | Short chain fatty acid concentrations in pore water of plant-soil microcosms, during the late vegetative and reproductive plant stage. Different letters indicate significant difference (mean ± SD, n = 3).

Emission of methane occurred in all microcosms **(Figure 3.6)** and turned out to be stable during the whole reproductive plant stage. Methane emissions were far highest in microcosms based on digested sludge. Formation of methane from microcosms with mixed inoculum was slightly lower compared to the control and therefore higher than in rice paddy soil microcosms, which emitted methane within a range of about a third of the control.

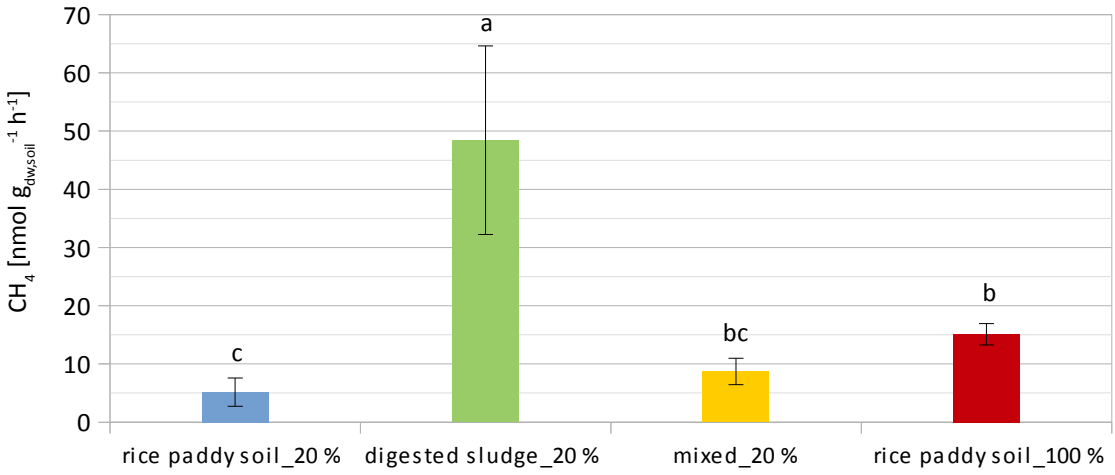

Figure 3.6 | Total emission of methane from plant-soil microcosms with different inocula during reproductive plant stage. Different letters indicate significant difference (mean ± SD, n = 3).

3.3 Translocation of plant-derived carbon in plant-soil microcosms

The translocation of plant derived carbon was followed by pulse-labeling of the plant-soil microcosms with $^{13}CO_2$ and measuring of ^{13}C values in the different carbon pools. After pulse-labeling with $^{13}CO_2$, concentration of CO_2 in the chamber's headspace was decreased by the presence of the rice plants until reaching stable values on average 3.53 ± 0.39 % within 8 h. In this time, plants of all microcosms consumed about 97.7 ± 0.8 % of the available $^{13}CO_2$ from the headspace.

The $^{13}CO_2$ was fixed by the rice plants via photosynthesis within the above ground plant biomass and afterwards allocated to the plant roots **(Figure 3.7)**. Subsequently ^{13}C labeled carbon compounds were assumed to be originating from rhizodeposition and degradation of rhizodeposits could be followed to the formation of methane. Enrichment of ^{13}C in the rice plants biomass was in all microcosms higher in the above ground plant biomass compared to the roots. The rhizospheric soil showed no enrichment of ^{13}C at all, indicating no cross labeling of other carbon pools by translocation of labeled soil organic matter. No significant differences in ^{13}C enrichment of above ground plant biomass, roots, or rhizospheric soil could be detected with respect to the different soil-systems.

Short chain fatty acids of the pore water like propionate and acetate, as well as CO_2, were assumed to be potential precursors for methane formation. Enrichment of pore water propionate and acetate in microcosms with mixed inoculum was lower compared to the other inocula, indicating a higher conversion of initial soil organic carbon than root derived carbon. Microcosms based on digested sludge showed a higher labeling of propionate and acetate than the mixed inocula samples. For pore water CO_2, both soil-systems were in the same range of ^{13}C enrichment in comparison to the 100 % soil control. Compared with the other soil-systems, microcosms based on synthetic rice paddy soil showed by far the highest enrichment of ^{13}C in the pore water substances of propionate, acetate and CO_2. The ^{13}C values of these microcosms were slightly lower for acetate compared with the control, while for propionate and CO_2 enrichment was far higher than in the control. Also the ^{13}C labeling of emitted methane was highest for microcosms based on synthetic rice paddy soil, while those of other microcosms were in the same range. No ^{13}C enrichment occurred for formate or pyruvate in any of the microcosms.

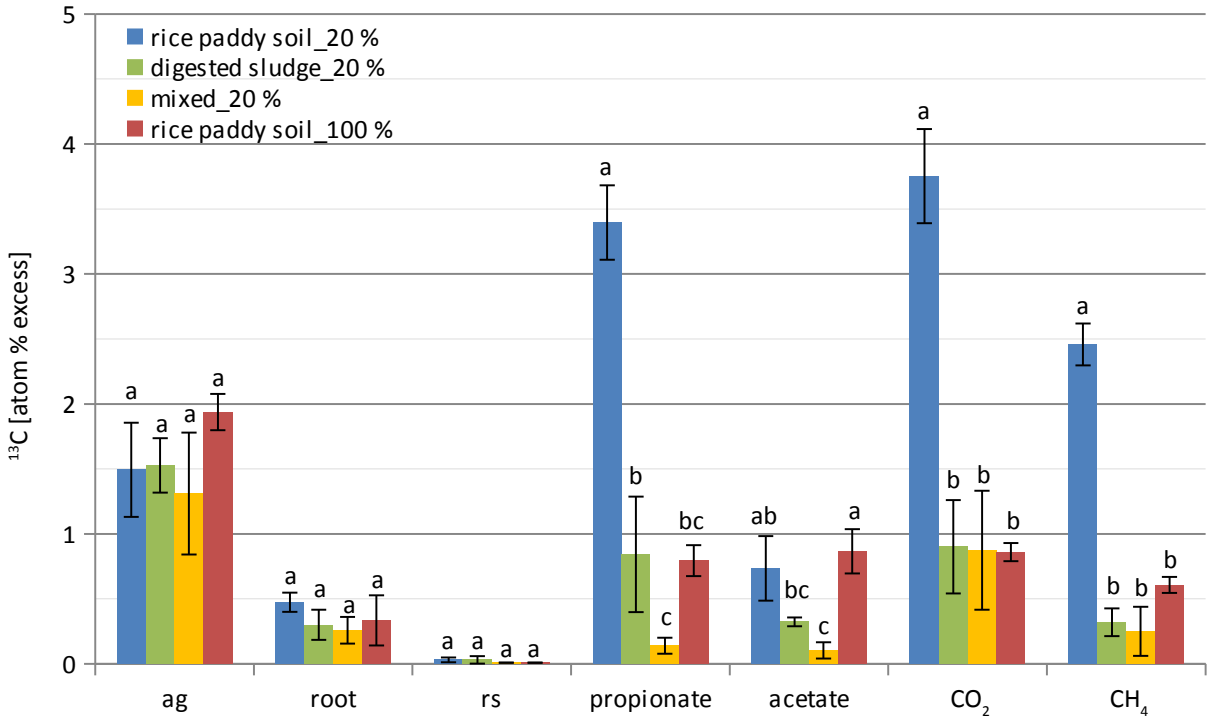

Figure 3.7 | Enrichment of ^{13}C in different carbon pools of plant-soil microcosms with different inocula, during the reproductive growth stage. These C-pools were above ground plant biomass (ag), plant roots, rhizospheric soil (rs), pore water substances of propionate, acetate, CO_2 as well as emitted CH_4. Different letters indicate significant difference within a carbon pool (mean ± SD, n = 3).

Beside the amount, ^{13}C enrichment rates (^{13}C enrichment per time) for propionate, acetate, CO_2, and CH_4 were also dependent on the soil-system of the different microcosms **(Figure 3.8)**. While labeling of propionate was at its maximum after 8 h in digested sludge and the mixed inoculum, it lasted 32 h in the 100 % soil control and 80 h in the rice paddy soil microcosms to reach the maximum state. Nevertheless, ^{13}C enrichment rates of propionate were higher for rice paddy soil and digested sludge compared with mixed inoculum and the control. The same enrichment time of 8 h could be determined for pore water CO_2 of digested sludge and mixed inoculum, while maximum labeling was reached after 56 h in the rice paddy soil microcosms and 80 h in the control. The ^{13}C enrichment rates for CO_2 were within the same range for all soil-systems and higher compared to the control. Maximum labeling of acetate occurred after 8 h in rice paddy soil, mixed inoculum and the control, while the maximum was reached after 32 h in digested sludge. For acetate, ^{13}C enrichment rates were higher in rice paddy soil and the control compared to digested sludge and mixed inoculum. The ^{13}C enrichment of emitted CH_4 was at its maximum after 32 h for

all soil-systems, in comparison to the control where it lasted 80 h to reach maximum labeling. The $^{13}CH_4$ enrichment rate for microcosms based on rice paddy soil was shown to be higher compared with the other soil-systems and the control.

Summarizing for all soil-systems, the measured ^{13}C enrichment rates indicated that microbial conversion of rhizodeposits to propionate occurred at higher rates in microcosms based on rice paddy soil and digested sludge, while conversion to CO_2 and CH_4 occurred most rapidly in rice paddy soil. The ^{13}C values of CO_2 and CH_4 in rice paddy soil microcosms were stable after enrichment, indicating some availability of rhizodeposits in the preceding carbon pools. The same could be detected for propionate, acetate, CO_2, and CH_4 in the control.

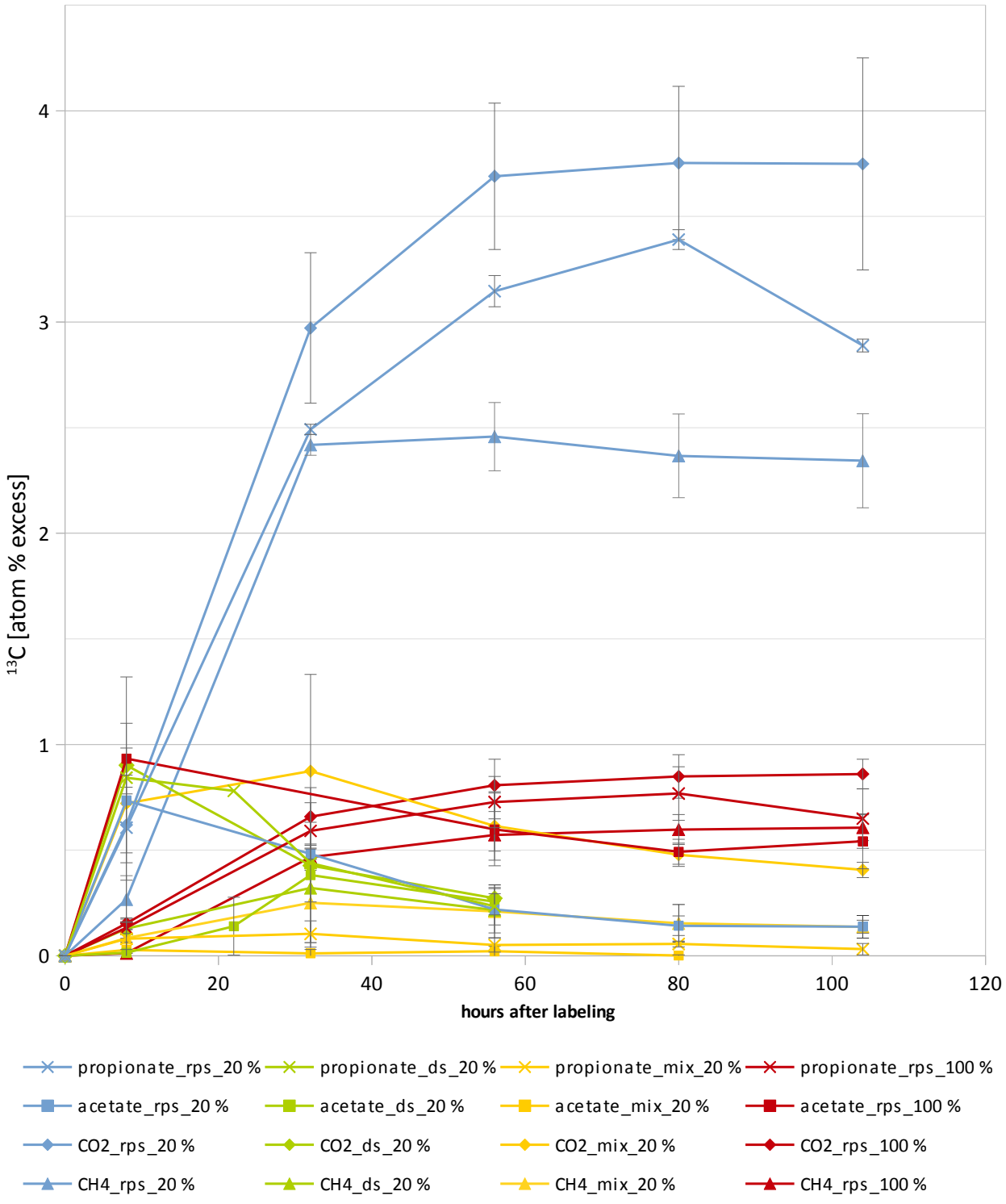

Figure 3.8 | Enrichment of ^{13}C in carbon pools of the pore water as well as the emitted methane, of microcosms with different soil-systems (**rps**: rice paddy soil, **ds**: digested sludge, **mix**: mixed inoculum), set in relation to time after initial labeling.

The emission of $^{13}CH_4$ was assumed to be originating from recently plant-assimilated carbon. Since not the entire root released carbon was labeled, the emission of methane based on rhizodeposition might be underestimated by this presumption. However, $^{13}CH_4$ emission rates, as well as the contribution of recently plant-assimilated carbon to the emission of total methane R_m, was dependent on the soil-system **(Figure 3.9)**. Microcosms with soil-systems based on rice paddy soil and digested sludge showed highest $^{13}CH_4$ emission rates, while those of the mixed inoculum were lower compared to the 100 % soil control. For rice paddy soil microcosms, R_m was also higher than in other microcosms. Although digested sludge microcosms showed a high production of $^{13}CH_4$, as well as a high formation of total methane, R_m was lower in microcosms based on digested sludge compared to those with rice paddy soil. R_m of microcosms based on mixed inoculum was within the same range as digested sludge systems, whereas both showed R_m values below the control. Therefore, methane formed in microcosms with mixed inocula was almost entirely formed by conversion of initial soil organic carbon.

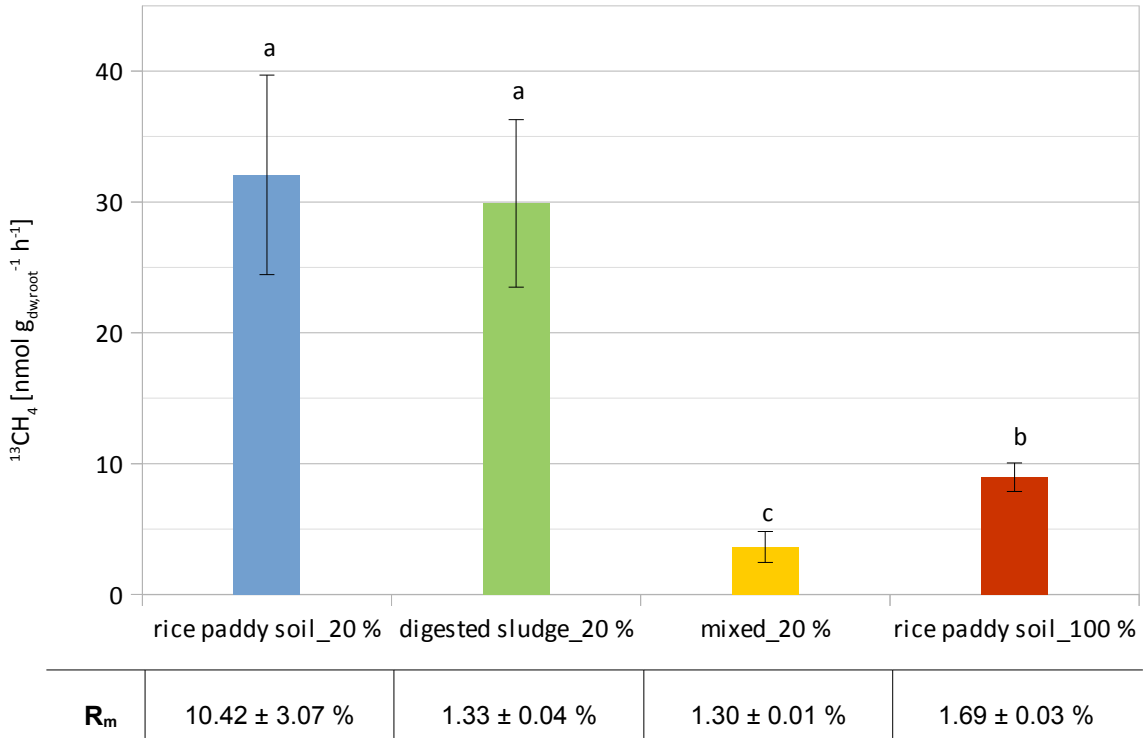

Figure 3.9 | Emission of methane originating from plant root derived carbon in plant-soil microcosms with different inocula during the time of highest ^{13}C-labeling of the methane pool. Different letters indicate significant difference (mean ± SD, n = 3). R_m: contribution of recently plant-assimilated carbon to total CH_4 emission.

3.3 Composition of the rhizospheric microbial community structure

Abundance and diversity of the microbial community was surveyed in rice-planted microcosms based on different soil-systems. Therefore, samples were divided between the different compartments of soil before planting, as well as rhizospheric soil and rice roots during the reproductive growth stage. Quantitative PCR was performed to determine abundance of methanogenic archaea and methane-oxidizing bacteria, as well as total bacteria and archaea. Using illumina MiSeq amplicon sequencing, the bacterial and methanogenic diversity and community composition was further evaluated.

3.3.1 Abundance of rhizosphere colonizing microorganisms

Copy numbers of archaeal and bacterial 16S rRNA genes, *mcrA*, and *pmoA* were quantitatively analyzed in microcosms with different soil-systems **(Figure 3.10)**. Before being planted with rice plants, soil of all soil-systems contained a number of archaeal 16S rRNA gene copies within the same range, which was slightly lower compared with the 100 % soil control. Archaeal abundance in the rhizospheric soil of digested sludge microcosms was considerably higher compared to the other soil-systems and within the same range as the control. Colonization of the rice roots by archaea occurred to the same extent in all soil-systems, but was about three times lower compared to the control. Copy number of bacterial 16S rRNA genes in unplanted soil was slightly higher in the soil-systems with digested sludge and mixed inoculum compared with rice paddy soil, which was within the same range as the control. Rhizospheric soil of microcosms with digested sludge and the control showed higher bacterial abundance than the other microcosms, while bacterial colonization of the rice root surface was highest for digested sludge and mixed inoculum.

The copy number of *mcrA* was slightly lower in microcosms based on synthetic rice paddy soil before planting compared to the other microcosms. The rhizospheric rice paddy soil also showed a slightly lower number of *mcrA* genes compared with the mixed inoculum, while those of digested sludge microcosms was about five times higher. Colonization of root surface by methanogenic archaea was within the same range for all microcosms based on synthetic soil-systems and about three times below that of the control.

The *pmoA* gene copy number in the unplanted soil was highest in soil-systems based on digested sludge. Colonization of rhizospheric soil and root surface by methane-oxidizing bacteria was also highest in digested sludge microcosms. Whereas abundance of *pmoA* in the rhizospheric soil was slightly higher for microcosms with rice paddy soil compared to those with mixed inoculum, both soil-systems showed *pmoA* gene copy numbers within the same range for root samples and in unplanted soil. Colonization of the root surface by methane-oxidizing bacteria occurred to the same extent in microcosms based on rice paddy soil, mixed inoculum and control.

Important parameters for microbial colonization are different between soil and root samples of the same mass. In order to compare microbial colonization of these different compartments, gene copy number of *mcrA* was set in relation to those of archaeal 16S rRNA genes and *pmoA* gene copy number in relation to bacterial 16S rRNA genes. An intense colonization of roots by methanogenic archaea could be detected in microcosms based on synthetic rice paddy soil. The rhizospheric of

these microcosms was also colonized by methanogenic archaea but within the same range of abundance as the soil before planting. For digested sludge, colonization of the rhizospheric soil by methanogenic archaea was intense, while abundance on the root surface was lower compared to the unplanted soil. Methanogenic abundance in the rhizospheric soil and the root samples of microcosms based on mixed inoculum was within the same range as before planting. Colonization by methanogens of the control microcosms occurred almost to the same extent as for the rice paddy soil-system, but abundance of methanogenic archaea in the rhizospheric soil was also higher compared to the unplanted soil within the control.

Distinct colonization by methane-oxidizing bacteria could be detected in the rhizospheric soil of all microcosms with synthetic soil-systems. For all these soil-systems, the relative number of *pmoA* was higher for rhizospheric soil compared with the unplanted soil. Colonization of root surface by methane-oxidizing bacteria occurred also in microcosms based on digested sludge, while for roots samples of the other soil-systems the abundance was within the same range as for soil before planting. The relative number of *pmoA* for rhizospheric soil and root samples of the control were lower compared to the soil before planting.

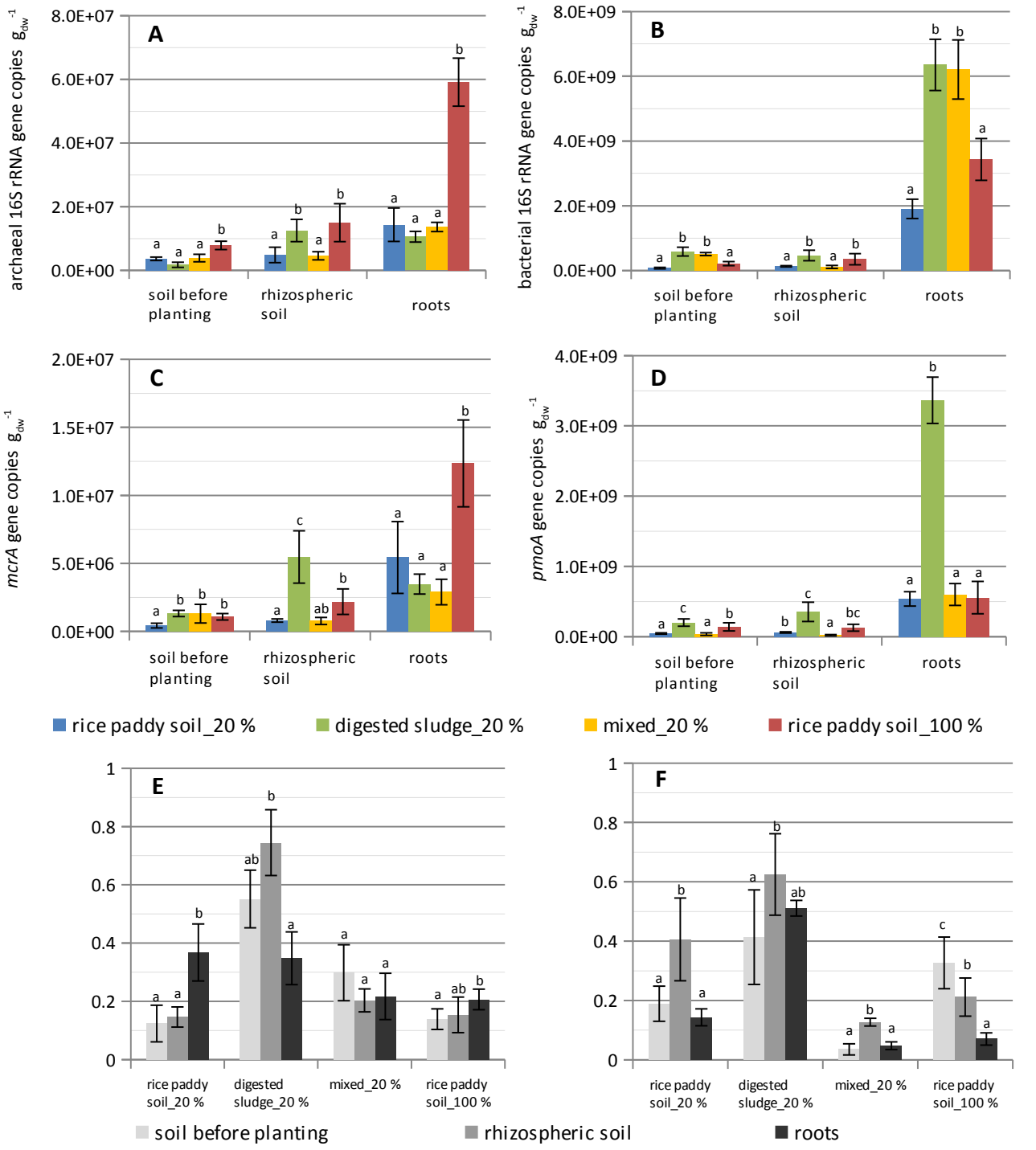

Figure 3.10 | Gene copy numbers quantified by qPCR of soil before planting, rhizospheric soil and roots during the reproductive plant stage from rice-planted microcosms with different soil-systems. Abundance of archaeal 16S rRNA genes **(A)**, bacterial 16S rRNA genes **(B)**, *mcrA* genes **(C)**, and *pmoA* genes **(D)**, as well as relative values of *mcrA* genes/archaeal 16S rRNA genes **(E)** and *pmoA* genes/bacterial 16S rRNA genes **(F)** for comparison of soil and root colonization. Different letters indicate significant difference (mean ± SD, n = 9) within either compartment (A to D) or microcosm (E and F).

3.3.2 Bacterial community structure in the rice rhizosphere

Bacterial 16S rRNA genes of the different compartments from the rice-planted microcosms were amplified with universal barcoded primers. For each compartment and soil-system we obtained at least 1,500 high-quality sequence reads **(Table 3.3)**. The number of reads and also the number of observed OTUs was different with respect to the different soil-systems. OTU- and read numbers in microcosms based on digested sludge were far lower compared to the other systems, as well as the species richness (Chao1 estimator). Nevertheless, predicted coverage (Good's coverage) and diversity (Shannon index) of digested sludge samples were only slightly lower in comparison to the other soil-systems. Furthermore, evenness (Pielou's evenness) revealed that the bacterial community composition in digested sludge was different to the microcosms. Only minor differences for sequencing results could be observed with respect to the different soil compartments.

Even samples with a high number of reads failed to cover the entire bacterial community. Nevertheless, coverage was at least 89 % in all microcosms and compartments. Sample coverage plotted against the number of bacterial species revealed that a higher number of reads would not result in a further increase in sample coverage in all compartments and microcosms except those based on digested sludge **(Figure 3.11)**.

Table 3.3 | Characteristics of 16S rRNA gene amplicon libraries of bacterial communities for different compartments of microcosms with different soil-systems.

compartment	soil-system	total no. of reads	no. of OTUs	coverage[a]	diversity index		
					richness[b]	diversity[c]	evenness[d]
soil before planting	rice paddy soil_20 %	84741	6928	0.95	7918	6.18	0.61
	digested sludge_20 %	1911	387	0.89	434	4.69	0.46
	mixed_20 %	187205	6957	0.98	7633	5.40	0.53
	rice paddy soil_100 %	124679	7186	0.97	7535	6.37	0.63
rhzispheric soil	rice paddy soil_20 %	70496	6234	0.95	6251	5.81	0.57
	digested sludge_20 %	2336	365	0.92	406	4.17	0.41
	mixed_20 %	43896	3250	0.96	3253	5.35	0.53
	rice paddy soil_100 %	74723	6416	0.96	6529	6.55	0.65
roots	rice paddy soil_20 %	184274	6624	0.98	7205	5.19	0.51
	digested sludge_20 %	1576	245	0.91	263	3.63	0.36
	mixed_20 %	134968	6022	0.97	6953	4.87	0.48
	rice paddy soil_100 %	118193	5734	0.98	6002	5.55	0.55

[a] Good's coverage estimator

[b] richness based on OTUs, Chao1 estimator

[c] diversity based on OTUs, nonparametric Shannon index

[d] Pielou's evenness based on OTUs

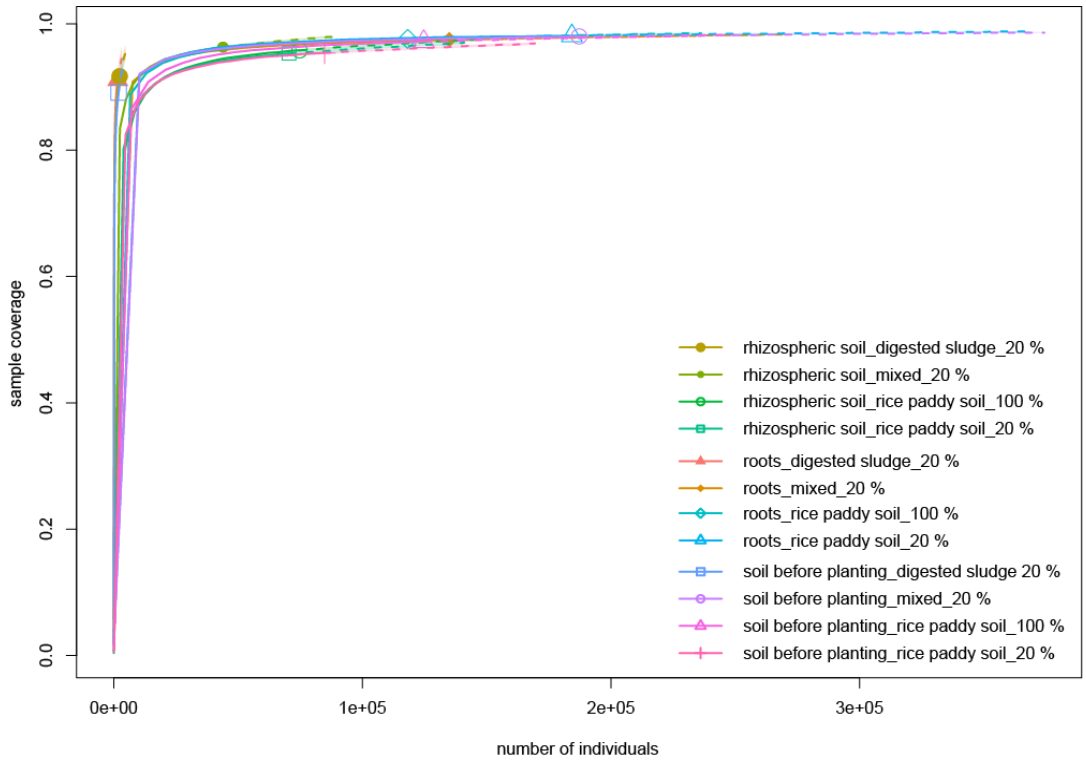

Figure 3.11 | Sample-sized-based rarefaction (solid lines) and extrapolation (dashed lines, up to twice as much as the reference sample size) of sample coverage, based on nonparametric Shannon index for 16S rRNA gene amplicon libraries of bacterial communities for different compartments of microcosms with different soil-systems.

Except for digested sludge systems, all microcosms contained the bacterial phyla of Proteobacteria, Firmicutes, Actinobacteria, Gemmatimonadetes, Chloroflexi, Deinococcus-Thermus, Bacteroidetes, Acidobacteria, and Planctomycetes **(Figure 3.12)**. Before planting with rice plants, Proteobacteria and Firmicutes were the dominant phyla within the bacterial communities of all soil-systems and the 100 % soil control. In all microcosms apart from those with digested sludge, rhizospheric soil showed a higher abundance of Proteobacteria than the soil before planting, while the abundance of Firmicutes in the rhizospheric soil was lower. Further, presence of Proteobacteria directly on the root surface was even higher, while these of Firmicutes was actually lower compared to the unplanted and rhizospheric soil. The phylum of Bacteroides was dominant in the rhizospheric soil of microcosms based on digested sludge and also highly abundant on rice roots. However, Proteobacteria represented the dominant phylum on the root surface in microcosms based on digested sludge as with the other microcosms.

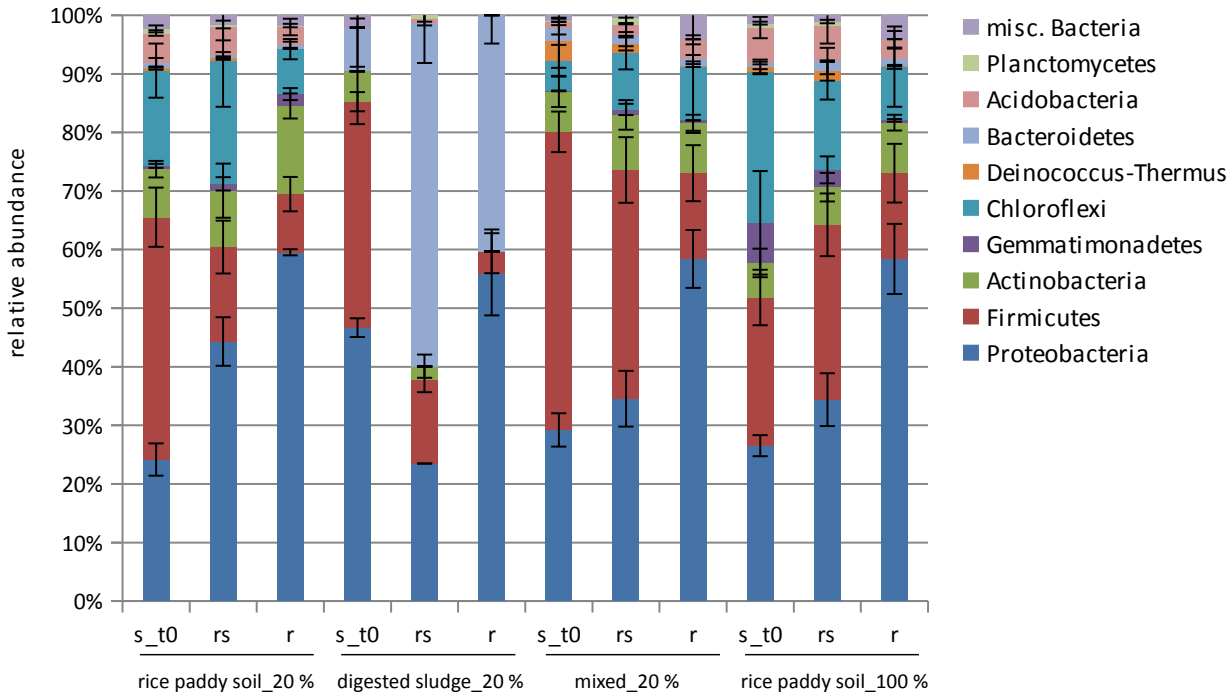

Figure 3.12 | Histograms of the relative abundance of bacterial phyla in soil before planting **(s_t0)**, as well as rhizospheric soil **(rs)** and root samples **(r)** during the reproductive plant growth stage, in rice-planted microcosms with different soil-systems obtained from illumina sequencing of bacterial 16S rRNA gene amplicons.

Bacterial communities of different soil-systems and soil compartments also differed at OTU-level **(Figure 3.13)**. While communities of unplanted soil and root surface in rice paddy soil microcosms showed some similarities to those of the 100 % soil control, microcosms based on digested sludge and mixed inoculum respectively possessed a distinct bacterial community composition. Only microcosms based on mixed inoculum showed some similarities between the rhizospheric soil community structure and those of the root surface and unplanted soil. Further, the samples with mixed inoculum clustered between microcosms based on rice paddy soil and digested sludge.

In addition, the rhizospheric community structure was surveyed in more detail via canonical correspondence analysis (CCA), considering several biotic and abiotic parameters. It could be shown that neither plant factors like plant height and plant weight, nor organic carbon content of the different soil-systems, had an effect on bacterial community structure of the rice rhizosphere.

The amount of ^{13}C labeling for CO_2 and acetate, as well as $^{13}CH_4$ emission rate, showed a positive correlation with the rhizospheric community composition. With respect to these considered parameters, bacterial communities of rhizospheric soil from digested sludge shared some similarities with those of the rhizospheric soil and root surface from mixed inoculum, while all other communities differed from each other. All other microcosms beside those with mixed inoculum showed a distinct separation of bacterial community composition of the rhizospheric soil and the root surface. Ordination showed resemble patterns when calculated without digested sludge samples **(Figure S3.1)** considering that clustering was not biased by their lower diversity.

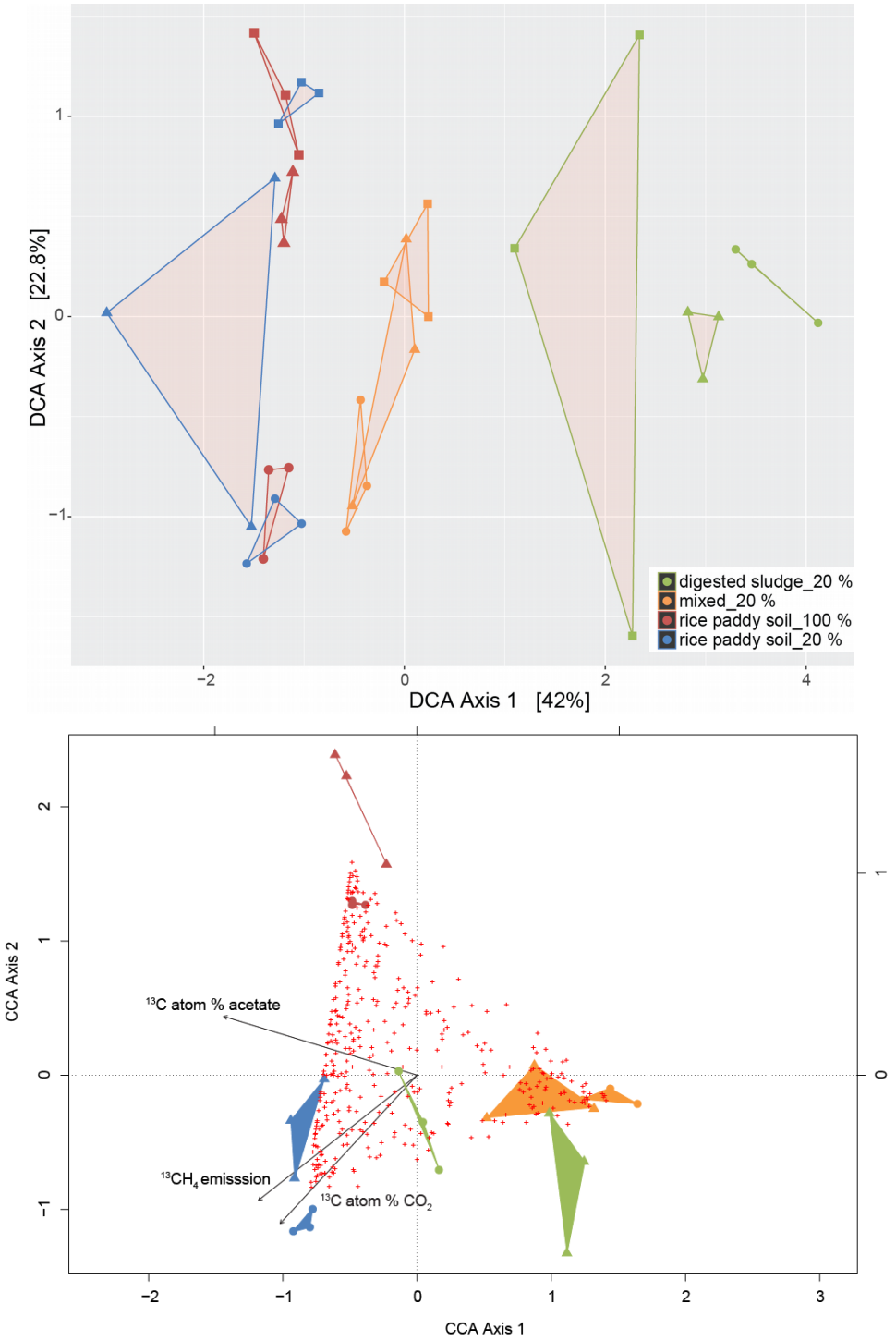

Figure 3.13 | Phylogenetic patterns in the bacterial community structure of rice-planted microcosms with different soil-systems, during the reproductive growth stage. Community similarities were calculated based on OTU-level. **Above:** Ordination by DCA for all samples. **Below:** CCA for rhizospheric soil and root samples. Arrows indicate the direction and relative importance (arrow length) of environmental variables associated with bacterial community structures respectively. Solely the environmental variables significantly influencing the model were displayed (ANOVA $p < 0.05$). Symbols indicate different compartments of the microcosms: soil before planting (■), rhizospheric soil (▲), and root samples (●), or bacterial OTUs (+).

Combining CCA with an indicator species analysis revealed the identity of OTUs whose abundance was positively correlated to the emission of $^{13}CH_4$. Most of the OTUs linked to $^{13}CH_4$ emission could be assigned to the phyla of Proteobacteria and Firmicutes **(Figure 3.14)**. Within the Proteobacteria, OTU_3 (*Anaeromyxobacter*) was highly abundant in rhizospheric soil and root samples of rice paddy soil microcosms, as well as in the rhizospheric soil of microcosms based on mixed inoculum and on roots within the 100 % soil control. In all of these microcosms, OTU_3 was far more abundant in the rhizospheric soil or on the root surface compared to the unplanted soil. Nevertheless, OTU_3 was totally absent in digested sludge microcosms. Furthermore, OTU_4 (*Anaeromyxobacter*) and OTU_8 (*Bradyrhizobium*) were highly abundant on root samples in the rice paddy soil-system. In microcosms based on mixed inoculum, OTU_8 was more frequent in the rhizospheric soil and the root surface than in soil before planting. All other OTUs within the Proteobacteria showed to be highest abundant in rice paddy soil-system, especially on the root surface of these microcosms.

Within the Bacteroidetes, OTU_160 (*Bacteroidales*, BA008) was the only OTU linked to $^{13}CH_4$ emission and could only be detected in microcosms based on digested sludge. Within these microcosms, OTU_160 was frequent in rhizospheric soil and even more abundant on the root surface. Also at phylum-level, only digested sludge microcosms showed a distinctive colonization by members of the Bacteroidetes in the total rhizosphere. Nevertheless, besides OTU_160 all other OTUs linked to emission of $^{13}CH_4$ were absent in the rhizosphere of digested sludge microcosms.

OTUs linked to $^{13}CH_4$ emission within the phyla of Firmicutes, Gemmatimonadetes, Fibrobacteres, Cyanobacteria, and Chloroflexi were of low abundance in rhizospheric soil and root samples of all microcosms. Apart from OTU_29 (*Desulfosporosinus*) and OTU_41 (*Pelotomaculum*), all other OTUs of these phyla showed no preference in colonizing the rhizospheric soil or the root surface, in comparison to the unplanted soil. In contrast, OTU_5 (*Kineosporiaceae*) within the Actinobacteria showed a high abundance on the root surface in rice paddy soil as well as the control.

Analysis on OTU-level revealed that the bacterial community linked to emission of $^{13}CH_4$ in the overall rhizosphere of microcosms based on digested sludge was far different from those of the other soil-systems **(Figure 3.15)**. Since digested sludge showed a unique composition of the bacterial rhizospheric community linked to $^{13}CH_4$ emission, no ubiquitous bacterial OTUs could be detected which were present in the rhizosphere of all microcosms. Nevertheless, OTU_3 (*Anaeromyxobacter*), OTU_4 (*Anaeromyxobacter*), OTU_97 (*Anaeromyxobacter*),

OTU_8 (*Bradyrhizobium*), OTU_33 (*Clostridium*), and OTU_5 (*Kineosporiaceae*) were ubiquitously present in the rhizospheric soil and on the root surface in rice paddy soil and mixed inocula microcosms, as well as in the 100 % soil control.

Bacterial composition of the rice-planted microcosms changed in abundance with respect to the different soil compartments **(Figure 3.16)**. OTU_1 (*Rickettsiales*), OTU_4 (*Anaeromyxobacter*), OTU_5 (*Kineosporiaceae*), OTU_8 (*Bradyrhizobium*), OTU_10 (*Pleomorphomonas*), and OTU_12 (*Methylosinus*) showed to be characteristic for rice roots. Only OTU_19 (*Azospirillum*) was prominent in the rhizospheric soil, while OTU_3 (*Anaeromyxobacter*) and OTU_14 (*Myxococcales*) were characteristic of the rhizosphere in total. Primarily representatives of *Clostridiaceae* (OTU_2, OTU_15, OTU_16, and OTU_33) were strongly associated with rhizospheric soil or soil before planting, instead of the root surface. Only OTU_22 and OTU_20 were consistently represented among all soil compartments.

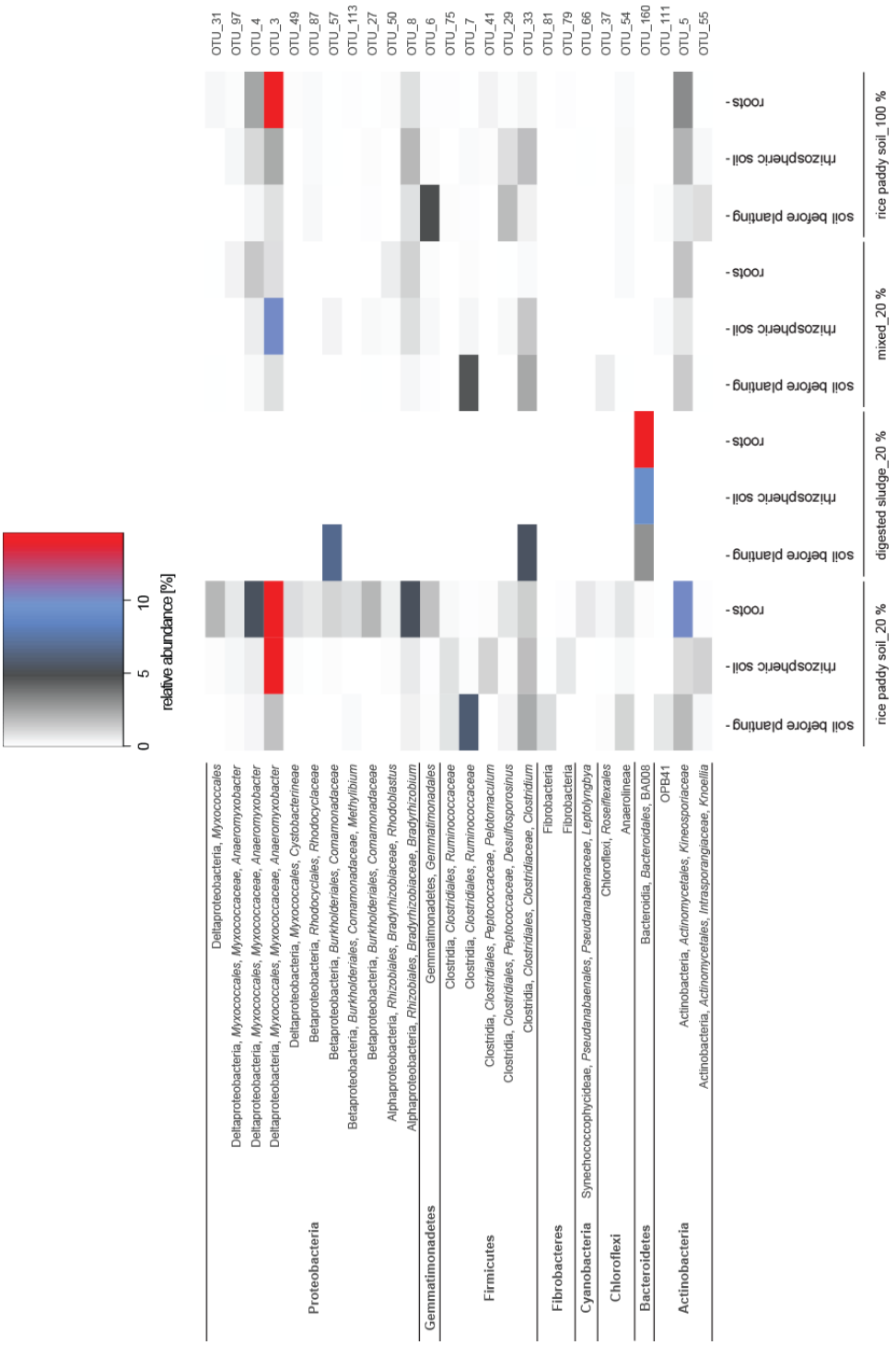

Figure 3.14 | Relative abundance of bacterial OTUs, linked to the emission of $^{13}CH_4$. These OTUs were selected from the indicator species of the CCA **(Figure 3.13)**, responsible for clustering along $^{13}CH_4$ emission. Only OTUs which showed a relative abundance in average of at least 0.5 % in one soil compartment of microcosms based on a different soil-system have been selected. Phylogeny is shown to the lowest taxonomic rank which could be assigned.

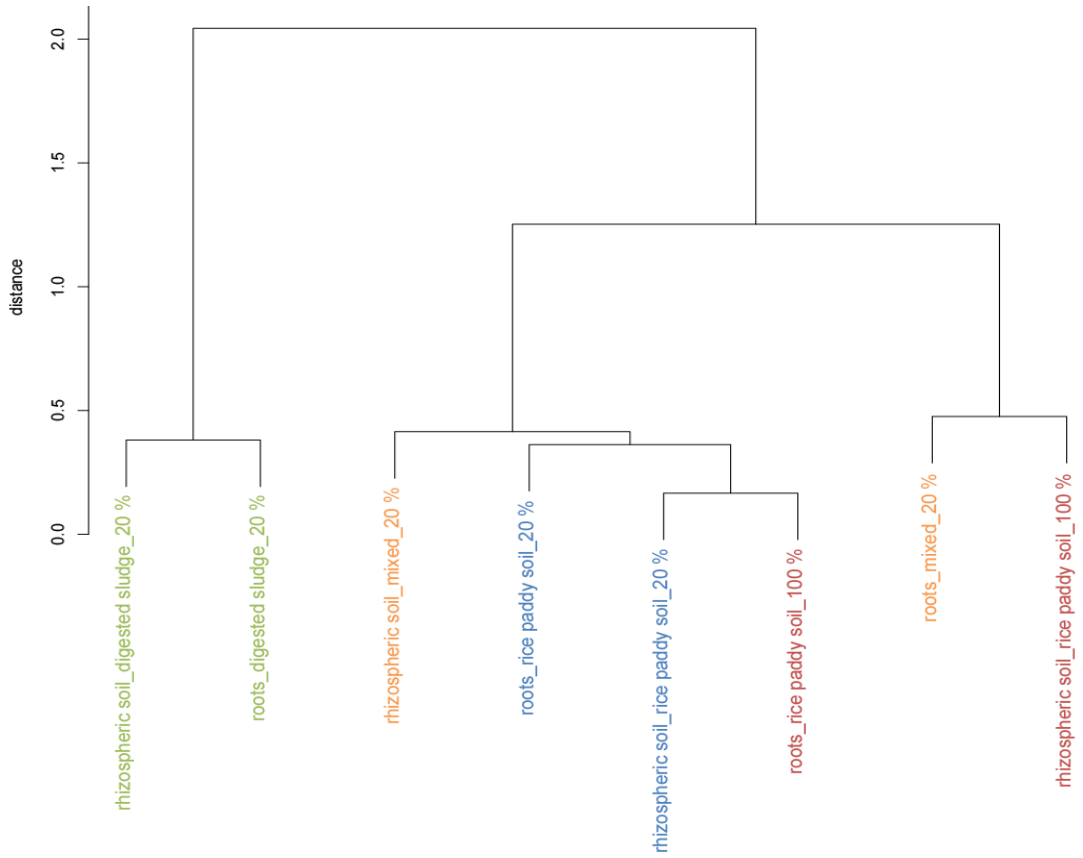

Figure 3.15 | Cluster analysis of the bacterial community in the overall rhizosphere of different soil-systems, linked to $^{13}CH_4$ emission. The samples are clustered according to Bray-Curtis distances based on the relative abundance of bacterial OTUs linked to the emission of $^{13}CH_4$.

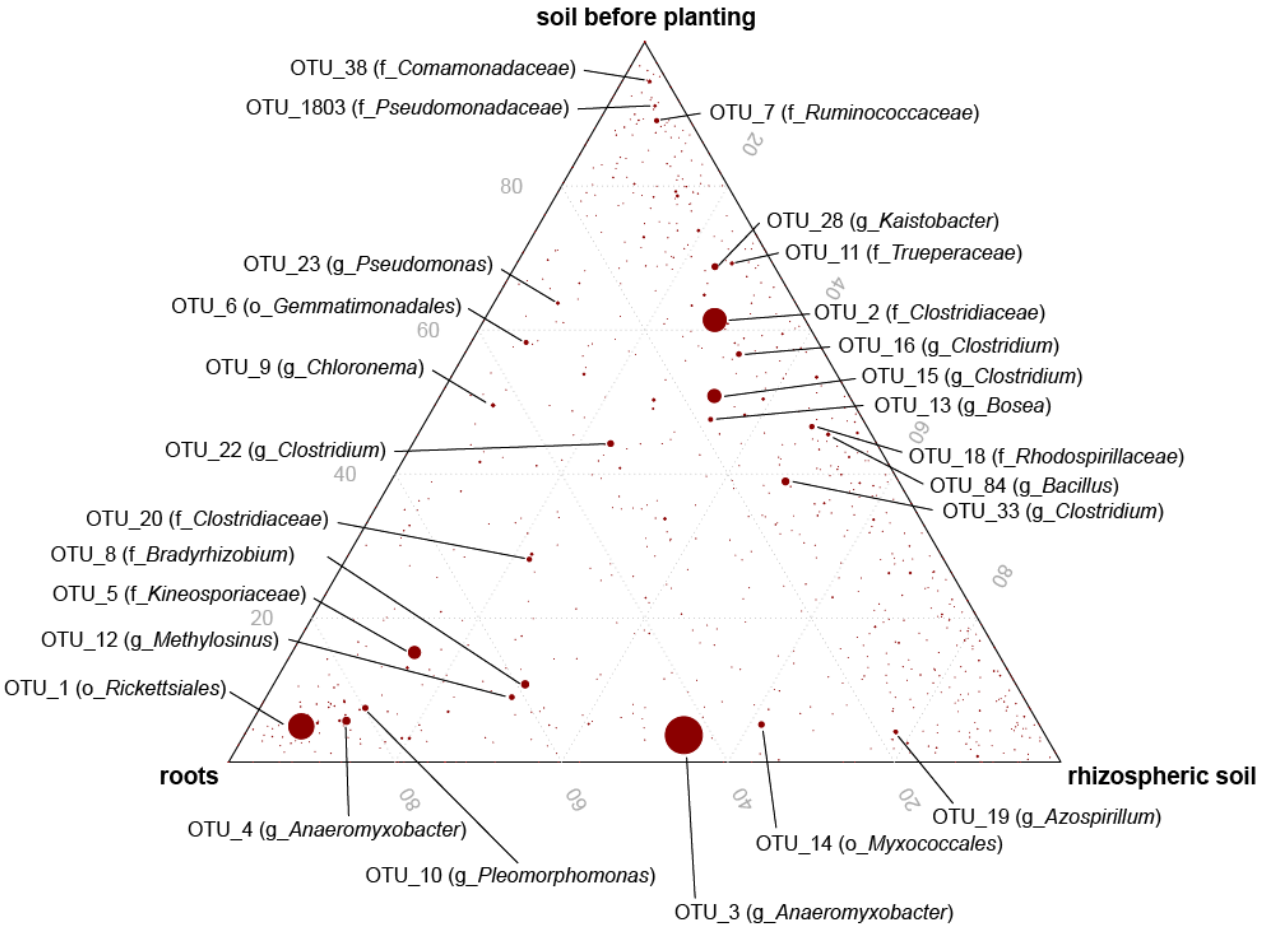

Figure 3.16 | Ternary plot of the distribution of bacterial OTUs across the different soil compartments of the rice-planted microcosms. Respective circle size represents the relative abundance of the OTU in the entire data set over all microcosms (as reference, OTU_3 = 8 %), the position specifies the average abundance in the respective soil compartment. Axes represent the percentage of reads associated with each sample for each OTU. Phylogeny is shown to the lowest taxonomic rank which could be assigned (o: order, f: family, and g: genus).

3.3.3 Methanogenic community structure in the rice rhizosphere

The *mcrA* genes in different compartments of the rice-planted microcosms were amplified with universal barcoded primers. At least 6,000 high-quality sequence reads were obtained for each compartment and soil-system **(Table 3.4)**. The number of reads and also the number of observed OTUs were different with respect to the different soil-systems. Read numbers of microcosms based on digested sludge were lower compared to the other soil-systems. Only minor differences could be observed for number of OTUs, predicted coverage (Good's coverage), species richness

(Chao1 estimator), and diversity (Shannon index) between different soil-systems and soil compartments. However, all samples revealed at least 99 % of the methanogenic community composition according to the sample coverage. Sample coverage plotted against the number of methanogenic species showed that a higher number of reads would not result in a further increase in the sample coverage in all compartments and microcosms **(Figure 3.17)**.

Table 3.4 | Characteristics of *mcrA* gene amplicon libraries of methanogenic communities for different compartments of microcosms with different soil-systems.

		total no. of			diversity index		
compartment	**soil system**	**reads**	**no. of OTUs**	**coverage**[a]	**richness**[b]	**diversity**[c]	**evenness**[d]
soil before planting	rice paddy soil_20 %	40347	24	0.99	21.83	1.79	0.53
	digested sludge_20 %	8236	22	0.99	19.61	1.74	0.51
	mixed_20 %	46892	26	1.00	21.78	2.01	0.59
	rice paddy soil_100 %	53886	26	0.99	23.61	1.94	0.58
rhzospheric soil	rice paddy soil_20 %	11857	23	0.99	22.17	1.82	0.54
	digested sludge_20 %	7654	20	0.99	16.33	1.55	0.45
	mixed_20 %	14436	26	0.99	26.44	1.88	0.56
	rice paddy soil_100 %	62000	23	1.00	20.67	1.98	0.59
roots	rice paddy soil_20 %	61505	22	1.00	20.17	1.71	0.50
	digested sludge_20 %	6198	20	0.99	26.33	1.60	0.47
	mixed_20 %	67539	26	1.00	23.00	2.21	0.65
	rice paddy soil_100 %	7998	19	1.00	19.67	1.83	0.54

[a] Good's coverage estimator

[b] richness based on OTUs, Chao1 estimator

[c] diversity based on OTUs, nonparametric Shannon index

[d] Pielou's evenness based on OTUs

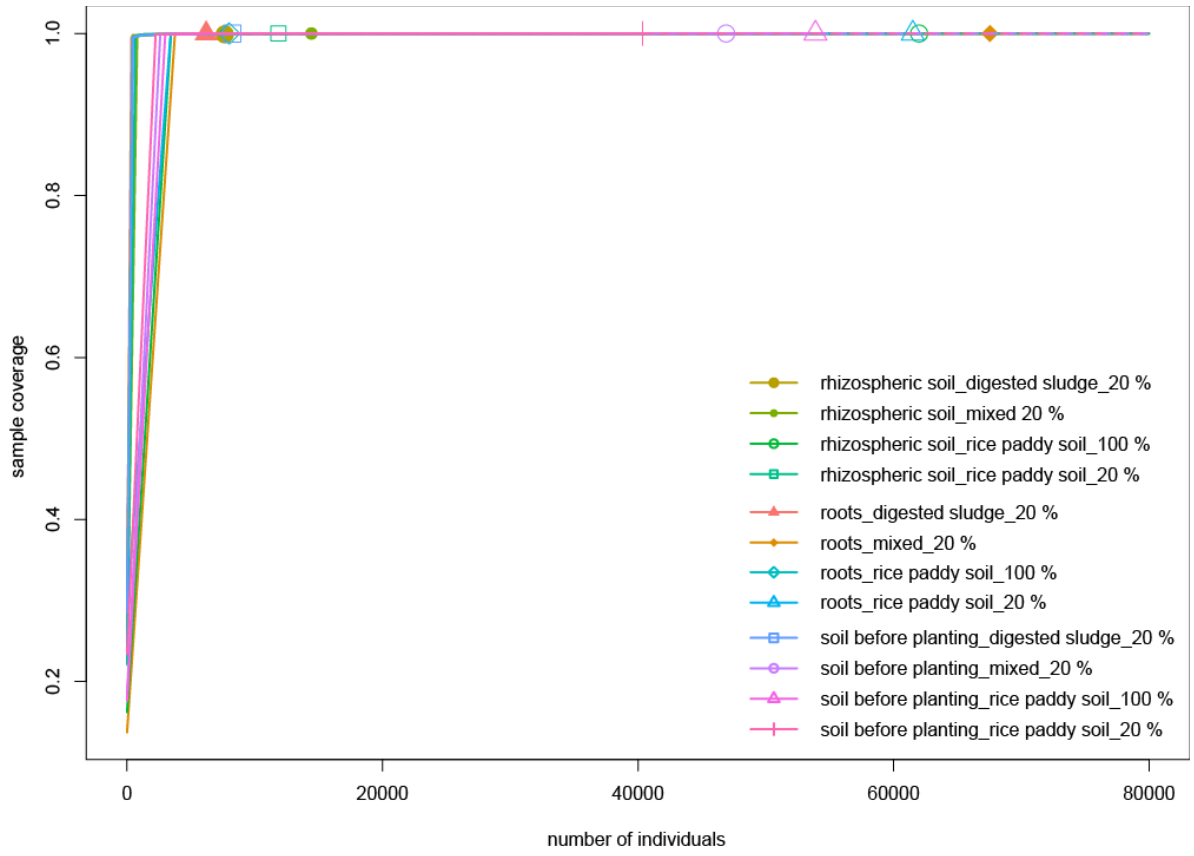

Figure 3.17 | Sample-sized-based rarefaction (solid lines) and extrapolation (dashed lines, up to twice as much as the reference sample size) of coverage based on nonparametric Shannon index for *mcrA* gene amplicon libraries of methanogenic communities for different compartments of microcosms with different soil- systems.

The community composition of methanogenic families strongly differed between soil compartments with respect to the different soil-systems **(Figure 3.18)**. Except in the digested sludge system all microcosms contained the methanogenic families of *Methanobacteriaceae*, *Methanosaetaceae*, *Methanosarcinaceae*, *Methanocellaceae*, and *Methanoregulaceae*, while *Methanomassiliicoccaceae* and *Methanomicrobiaceae* could only be detected at low abundances in some of the samples.

The unplanted rice paddy soil-system mainly contained *Methanosarcinaceae*, but also high numbers of *Methanocellaceae*, *Methanobacteriaceae*, and *Methanosaetaceae*. Rhizospheric rice paddy soil was primarily colonized by *Methanosarcinaceae* as well, but also with a high appearance of *Methanosaetaceae*, while abundance of *Methanocellaceae* was lower compared to the unplanted soil. Root surfaces of plants in rice paddy soil microcosms were mainly colonized by

Methanobacteriaceae and *Methanocellaceae*. Abundance of *Methanosaetaceae* was far lower on the root surface in comparison to the rhizospheric and unplanted soil of rice paddy soil microcosms.

Methanobacteriaceae was the dominant methanogenic family in all soil compartments of digested sludge microcosms. *Methanosaetaceae* only showed some minor abundance in the rhizospheric soil, and *Methanosarcinaceae* on the root surface of microcosms based on digested sludge.

Before planting, microcosms based on the mixed inoculum soil-system mainly contained *Methanobacteriaceae*, but also a considerable amount of *Methanosaetaceae*, *Methanoregulaceae*, and *Methanosarcinaceae*. The rhizospheric soil of these microcosms was mainly populated by *Methanosarcinaceae*, while *Methanobacteriaceae* were prevalent on the plant root surface. Compared with the rhizospheric methanogenic communities of other soil-systems, a considerable amount of *Methanomassiliicoccaceae* could be detected in the rhizospheric soil, as well as a high abundance of *Methanoregulaceae* on the root surface of the mixed inocula microcosms.

The methanogenic community composition in all compartments of the 100 % soil control microcosms was also different from those of the synthetic soil-systems. Whereas the community structure of the rhizospheric soil was similar to the unplanted soil, *Methanobacteriaceae* were primarily colonizing the root surface. Dominance of *Methanobacteriaceae* on the root surface of the control was analogous to the synthetic soil-systems, while the remaining methanogenic root community was different.

Analysis on family-level revealed that the methanogenic community structure of the root surface in all microcosms was clearly dominated by colonization of *Methanobacteriaceae*, regardless of the soil-system. Furthermore, the rhizospheric soil of all microcosms, except for these based on digested sludge, was mainly colonized by *Methanosaetaceae* and *Methanosarcinaceae*.

For consideration of the methanogenic diversity with a resolution beyond family-level, community structure was further analyzed on OTU-level **(Figure 3.19)**. Within the family of *Methanobacteriaceae*, OTU_3 and OTU_8 (*Methanobacterium*) showed to be the most frequent OTUs of the rhizospheric community. OTU_3 was highly abundant on the root surface in all microcosms except those based on mixed inoculum. Even its abundance was lower for the mixed inoculum soil-system, presence on the root surface of OTU_3 was higher compared to the rhizospheric and unplanted soil of mixed inocula microcosms. Besides the root samples, abundance of OTU_3 was also high in the rhizospheric soil of microcosms based on digested

sludge. OTU_6 (*Methanobacterium*) also showed a strong colonization of the rice root surface, but only in microcosms based on mixed inoculum. OTU_8 (*Methanobacterium*) was frequent on the root surface of all microcosms, and also in the rhizospheric soil of digested sludge microcosms and the 100 % soil control. For microcosms based on mixed inoculum, abundance of OTU_8 in the rhizospheric soil was below those on the root samples, but distinctly higher compared to the unplanted soil. In contrast OTU_4 (*Methanobacterium*) and OTU_7 were highly abundant in the mixed inocula soil-system and slightly less abundant in digested sludge before planting, but showed little potential to colonize the rhizosphere in these microcosms.

Within the *Methanosaetaceae*, rhizospheric soil of rice paddy soil and control microcosms was strongly colonized by OTU_2 (*Methanosaeta*), while it showed only a slight presence on roots. OTU_2 was highly abundant in the unplanted soil-system with mixed inoculum, but showed little potential to colonize the rhizosphere in these microcosms.

OTU_11 (*Methanosarcina*) was the only representative within the family of *Methanosarcinaceae*. For microcosms based on rice paddy soil, mixed inoculum, and control, OTU_11 distinctly colonized the rhizospheric soil. Furthermore, OTU_11 was abundant on the root surface of mixed inocula microcosms, as well as the control. A slight colonization of the rhizosphere occurs for digested sludge, but with a higher presence at the root surface.

Within the *Methanocellaceae*, OTU_34 (*Methanocella*) colonized the rhizospheric soil and the root surface of rice paddy soil microcosms. OTU_34 was totally absent in digested sludge and showed only a slight affinity to rhizospheric soil of mixed inocula microcosms. Furthermore, OTU_34 was highly abundant on roots of the control, while the colonization of the rhizospheric soil occurred at a lower rate compared to the soil before planting.

Within the family of *Methanoregulaceae*, the root surface of mixed inocula microcosms were colonized by OTU_1 (*Methanoregula*) and those of the control by OTU_33 (*Methanoregula*). Furthermore, OTU_1 (*Methanoregula*) showed some distinct appearance in the rhizospheric soil of microcosms based on mixed inocula, but with lower abundance compared to the unplanted soil.

For *Methanomassiliicoccaceae*, OTU_22 (*Methanomassiliicoccus*) only showed considerable abundance in the rhizospheric soil of microcosms based on mixed inoculum and on the root surface of the control.

In general, rhizospheric soil of rice paddy soil-systems was mainly colonized by OTU_2 (*Methanosaeta*) and OTU_11 (*Methanosarcinaceae*), belonging to methanogenic archaea and able to perform acetoclastic methanogenesis. Rhizospheric rice paddy soil also showed presence of the hydrogenotrophic OTU_34 (*Methanocella*), but with minor abundance. Also the rhizospheric soil of microcosms based on mixed inoculum showed a high abundance of OTU_11 (*Methanosarcinaceae*), but appearance of OTU_2 (*Methanosaeta*) was low in these microcosms. In microcosms based on digested sludge, colonization of rhizospheric soil and root surface mainly occurred by OTU_3 and OTU_8 belonging to the genus of the hydrogenotrophic *Methanobacterium*. The surface of rice roots in all microcosms was also mainly colonized by these hydrogenotrophic OTUs of *Methanobacterium*, as well as OTU_1 (*Methanoregula*) in microcosms based on mixed inoculum.

Furthermore, distinct OTUs could be determined for their presence in the rhizosphere of all soil-systems. OTU_2 (*Methanosaeta*), OTU_3 (*Methanobacteriaceae*), OTU_8 (*Methanobacterium*) and OTU_11 (*Methanosarcina*) showed to be ubiquitous in the rhizospheric soil, as well as on the root surface of plants in all tested microcosms. OTU_34 (*Methanocella*) was present in the rhizospheric soil and OTU_305 (*Methanobacteriaceae*) on the root surface of all microcosms.

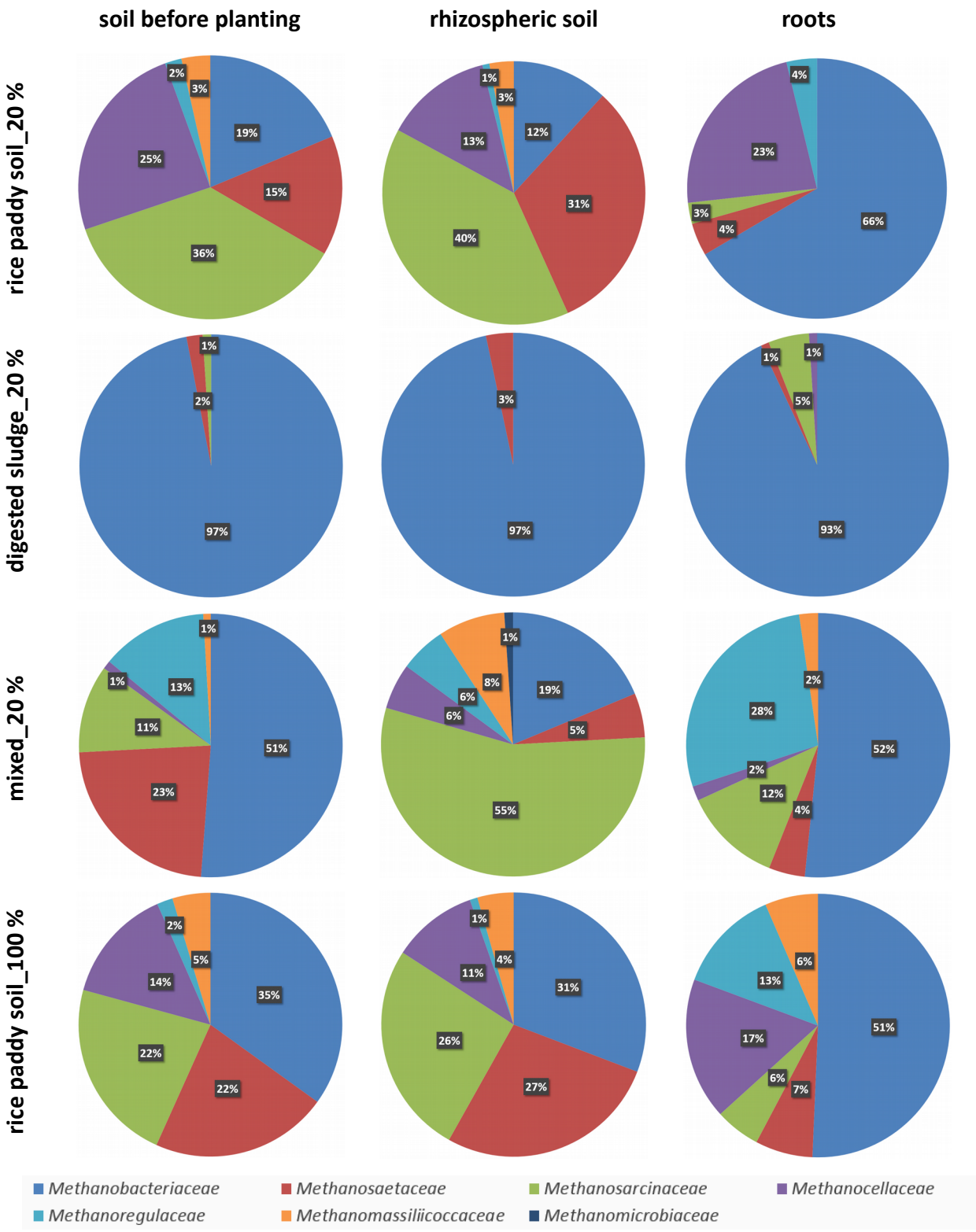

Figure 3.18 | Comparison of abundance of methanogenic archaea on family-level in soil compartments of planted-microcosms with different soil-systems during the reproductive plant growth stage.

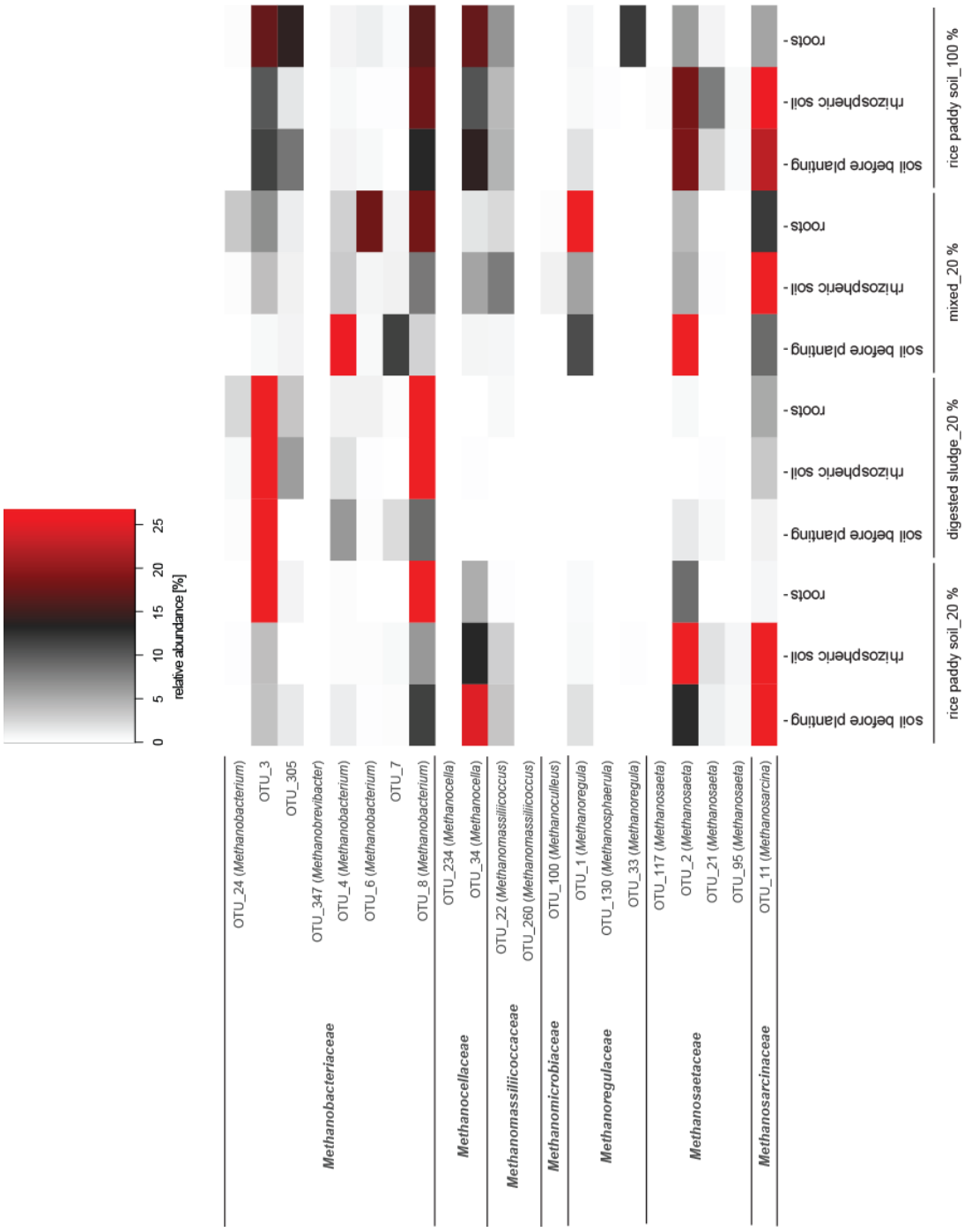

Figure 3.19 | Relative abundance of methanogenic archaea on OTU-level in soil compartments of planted-microcosms with different soil-systems during the reproductive plant growth stage. Phylogeny is shown to the lowest taxonomic rank which could be assigned.

Differences in the methanogenic community composition of the tested soil-systems and their compartments could also be confirmed by ordination on OTU-level **(Figure 3.20)**. Community structure in the rhizospheric soil of rice paddy soil, mixed inoculum, and the control shared some similarities and were also related to the rice paddy soil-system and control before planting. Digested sludge microcosms showed a distinct composition in the methanogenic community regardless of the compartment. Nevertheless, composition of rhizospheric soil and root samples of digested sludge were to be more related to each other than these before planting. The methanogenic community on the root surface of rice paddy soil microcosms showed some similarity to those of the control, while those of microcosms with mixed inocula showed a distinct composition.

Rhizospheric community structure of methanogenic archaea was observed in more detail via CCA, considering several biotic and abiotic parameters. It could be shown that neither plant factors like plant height and plant weight nor organic carbon content of the different soil-systems had an effect on the methanogenic community structure of the rhizosphere, whereas the amount of ^{13}C labeling for acetate, as well as $^{13}CH_4$ emission rate, showed a correlation with the rhizospheric community composition. With respect to the considered parameters, root colonizing methanogenic communities of rice paddy soil and digested sludge were more related to each other compared with the other root samples. Clustering of remaining samples occurred in more or less the same way without consideration of environmental factors.

Combining CCA with an indicator species analysis revealed that abundance of OTU_2 (*Methanosaetaceae*), OTU_3 (*Methanobacteriaceae*), OTU_8 (*Methanobacterium*), and OTU_34 (*Methanocella*) was positively correlated to the emission of $^{13}CH_4$. Relative abundance of the OTUs linked to formation of $^{13}CH_4$ was plotted and tested for significant differences **(Figure 3.21)**. Regardless of whether rhizospheric soil or root surface, both rice paddy soil and digested sludge microcosms showed the highest abundance of OTUs linked to $^{13}CH_4$ emission. For almost all of these OTUs, microcosms based on mixed inoculum showed a lower abundance in rhizospheric soil or on the root surface than the other soil-systems and the control, or were within the same range.

Cluster analysis on OTU-level revealed that the abundance of OTU_2 (*Methanosaetaceae*), OTU_3 (*Methanobacteriaceae*), OTU_8 (*Methanobacterium*), and OTU_34 (*Methanocella*) in the overall rhizosphere of digested sludge microcosms was related to the root communities of rice paddy soil and control microcosms **(Figure 3.22)**. The methanogenic community linked to $^{13}CH_4$

emission in the overall rhizosphere of mixed inocula microcosms was more related to the rhizospheric soil composition of rice paddy soil and control microcosms.

The methanogenic community composition of the rice-planted microcosms changed with respect to the different soil compartments **(Figure 3.23)**. OTU_1 (*Methanoregula*), OTU_3 (*Methanobacteriaceae*), OTU_6 (*Methanobacterium*), OTU_8 (*Methanobacterium*), OTU_24 (*Methanobacterium*), and OTU_33 (*Methanoregula*) were characteristic for the surface of the rice roots. OTU_11 (*Methanosarcina*), OTU_21 (*Methanosaeta*), OTU_22 (*Methanomassiliicoccus*), and OTU_95 (*Methanosaeta*) were prominent in rhizospheric soil, while OTU_4 (*Methanobacterium*) and OTU_7 (*Methanobacteriaceae*) seemed to be related to soil before planting. Only OTU_305 (*Methanobacteriaceae*) was consistently represented among all soil compartments, whereas OTU_2 (*Methanosaeta*) and OTU_34 (*Methanocella*) were characteristic for rhizospheric and unplanted soil.

Overall, hydrogenotrophic methanogens, predominately *Methanobacterium*/*Methanobacteriaceae*, preferred colonization of the root surface while methanogens able to perform acetoclastic methanogenesis primarily colonized the rhizospheric soil.

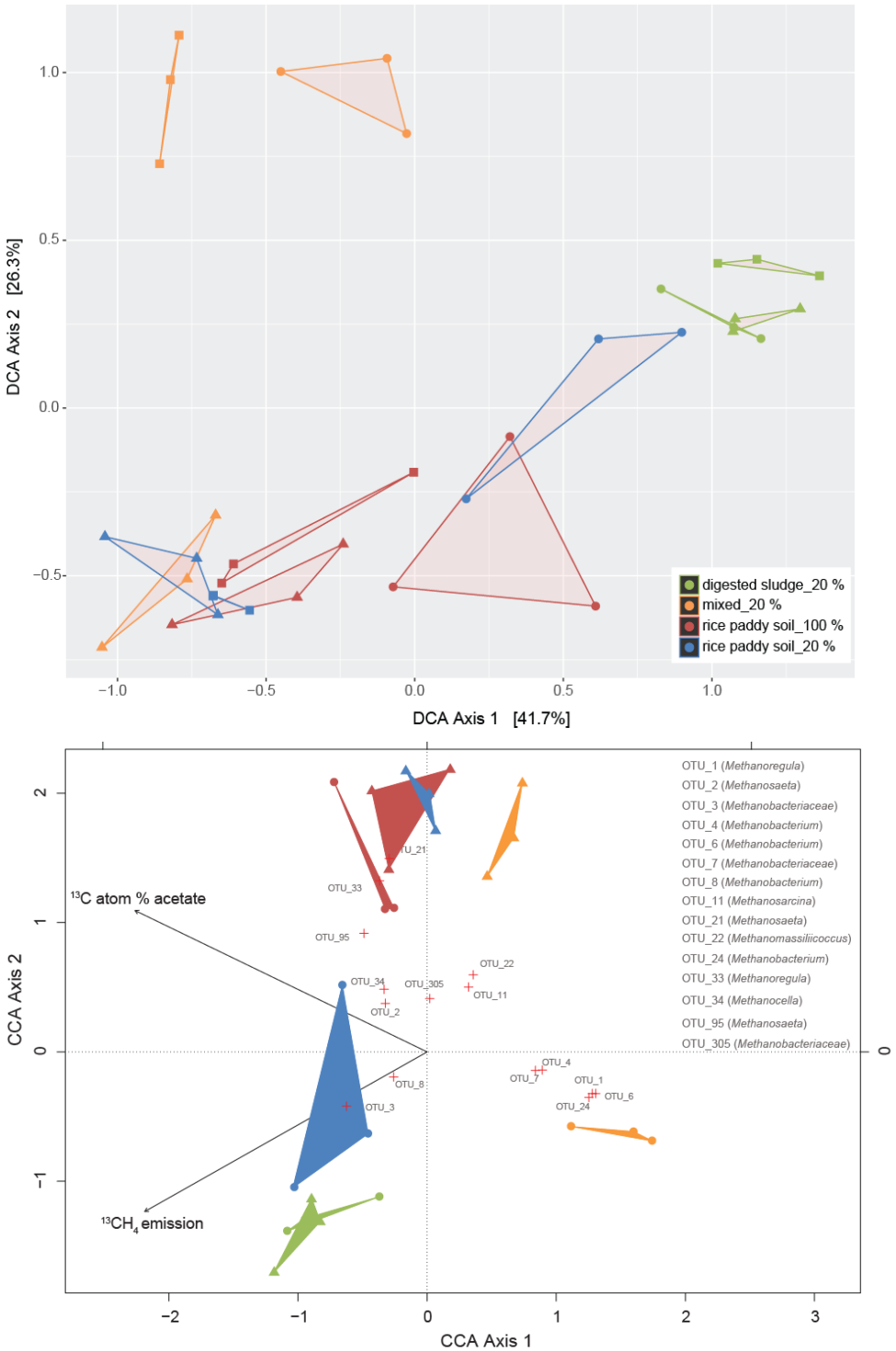

Figure 3.20 | Phylogenetic patterns in methanogenic community structure of rice-planted microcosms with different soil-systems, during the reproductive growth stage. Community similarities were calculated based on OTU-level. **Above:** Ordination by DCA for all samples. **Below:** CCA for rhizospheric soil and root samples. Arrows indicate the direction and relative importance (arrow length) of environmental variables associated with methanogenic community structures respectively. Solely the environmental variables significantly influencing the model were displayed (ANOVA $p < 0.05$). Symbols indicate different compartments of the microcosms: soil before planting (■), rhizospheric soil (▲), and root samples (●), or methanogenic OTUs (+).

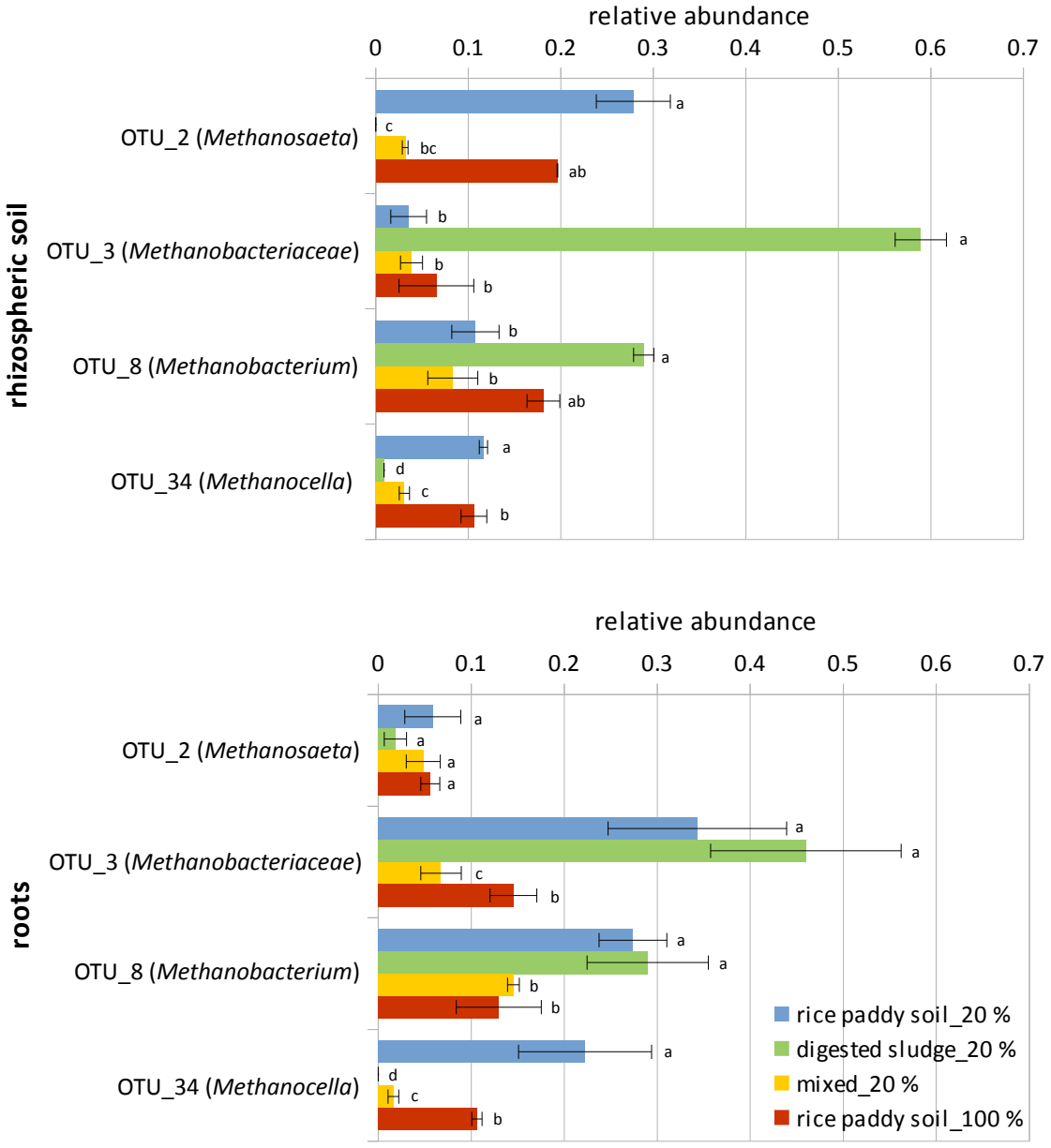

Figure 3.21 | Relative abundance of methanogenic OTUs linked to the emission of $^{13}CH_4$. These OTUs were selected from the indicator species of the CCA **(Figure 3.20)**, responsible for clustering along $^{13}CH_4$ emission. Phylogeny is shown to the lowest taxonomic rank which could be assigned. Different letters indicate significant difference within a OTU (mean ± SD, n = 3).

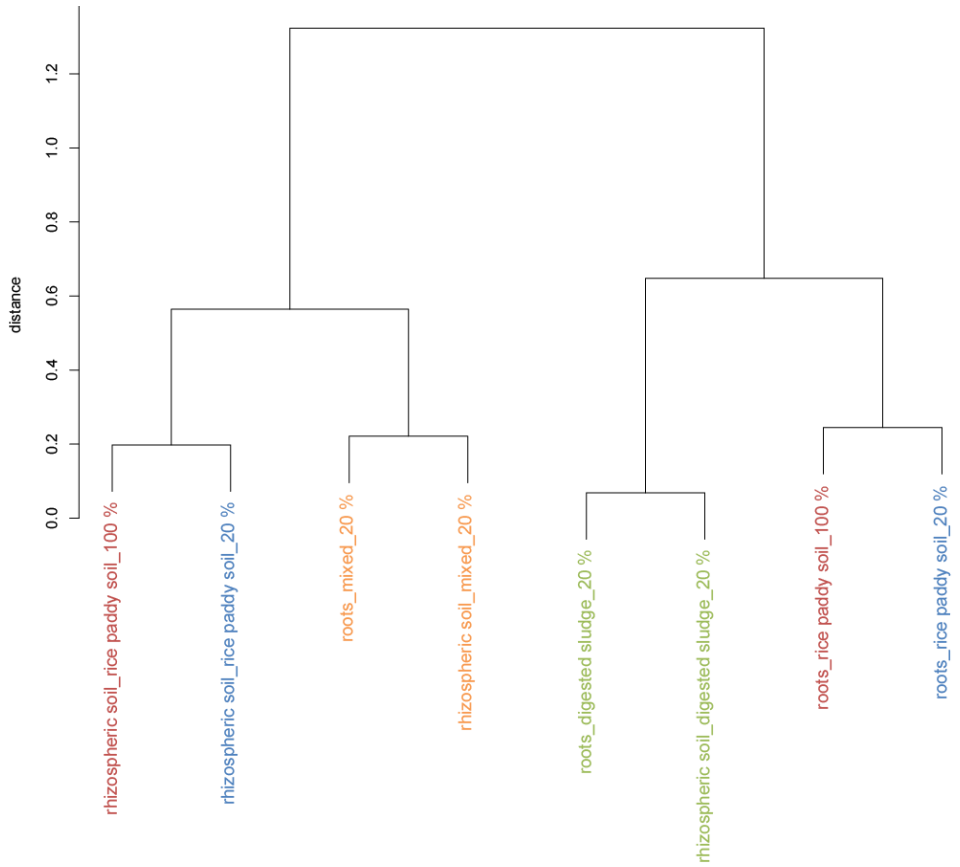

Figure 3.22 | Cluster analysis of the methanogenic community in the rhizosphere linked to $^{13}CH_4$ emission. The samples are clustered according to Bray-Curtis distances based on the relative abundance of OTUs linked to the emission of $^{13}CH_4$.

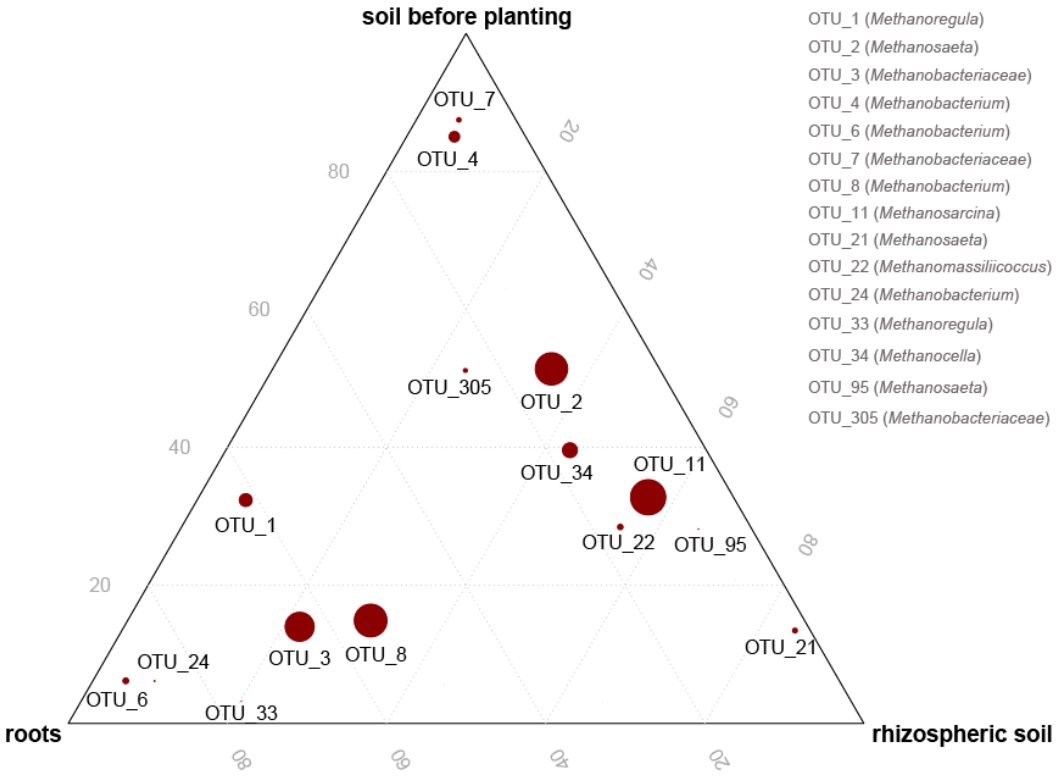

Figure 3.23 | Ternary plot of the distribution of methanogenic OTUs across the different soil compartments of the rice-planted microcosms. Respective circle size represents the relative abundance of the OTU in the entire data set over all microcosms (as reference, OTU_11 = 18 %), the position specifies the average abundance in the respective soil compartment. Axes represent the percentage of reads associated with each sample for each OTU. Phylogeny is shown to the lowest taxonomic rank which could be assigned.

IV. Discussion

4.1 Advancement of a plant-soil model system based on synthetic soil-systems with different inocula

The use of inoculation to manipulate the microbial rhizospheric community composition was initially intended to stimulate the plant beneficial rhizospheric microbial activity (Lynch, 1994). Recent studies also used this idea to investigate the role of plant derived carbon on carbon cycling in rice-plant microcosms based on rice paddy soil (Pump and Conrad, 2014; Pump et al., 2015). In this project we used inoculated synthetic soil-systems to provide different initial microbial communities for colonization of the rice rhizosphere, while simultaneously minimizing the abiotic factors of the different inocula.

In order to reach this goal, it was necessary to modify the sand-vermiculite amended microcosms of (Pump and Conrad, 2014) to establish reliable plant-soil model systems, which are usable with other inocula beside rice paddy soil. For this purpose we created synthetic soil-systems consisting of inert matrix (sand-vermiculite) to 80 % and of an environmental sample as an inoculum to 20 %. We also increased the volume of the soil-system in the microcosms to enable extensive colonization of the inert matrix by microorganisms of the inocula. To further improve the methanogenic activity in the system based on rice paddy soil, xylan and cellulose (1:1) were added in a concentration of 1.0 g kg^{-1}. Based on these soil-systems, plant-soil model systems could be successfully established. The use of such microcosms allowed rice plants to grow in the presence of different initial microbial communities. Furthermore, this approach enabled the investigation of the colonization of the rice roots, as well as the degradation of rhizodeposits to methane, on the basis of these initial communities. It has to be mentioned that experiments with microcosm in general may alter the root-soil relationship and therefore change the amount of belowground carbon translocation (Kuzyakov and Gavrichkova, 2010).

In order to further validate the reliability of these modified model-systems, we evaluated several parameters. The pH value of a soil affects all chemical, physical and biological soil properties and therefore also the microbial activity (Andersson and Nilsson, 2001; Wardle, 1992) and microbial community structure (Wardle, 1992) of the soil. However, microcosms based on the rice paddy- and mixed inoculum soil-system, as well as those based on the 100 % rice paddy soil control, showed no differences in the porewater pH value during the reproductive plant stage **(Figure 3.4)**. While pH values of all those microcosms were within a neutral soil pH- value, only the ones based on digested sludge reached a slightly alkaline value at the reproductive plant stage. According to (Fierer and Jackson, 2006), the pH value of different soil types largely explains differences in bacterial diversity. Root-mediated changes of the pH value also show a direct influence on growth and composition of the microbial rhizospheric community (Hinsinger et al., 2003). However, statistical analysis by ANOVA revealed that neither pH values nor soil organic carbon and nitrogen content of the different soil-systems could explain differences in bacterial **(Figure 3.13)** and methanogenic community composition **(Figure 3.20)** of the microcosms.

Plant growth in total was almost the same as in the unmodified plant-soil model system (Pump and Conrad, 2014). Even if plant height for rice-paddy soil- and digested sludge soil-systems were slightly lower compared to the other microcosms **(Figure 3.3)**, differences in plant height between these microcosms and the 100 % rice paddy soil control were in the same range as the original model system (Pump and Conrad, 2014). Furthermore, all microcosms based on synthetic soil-systems exhibited the same plant growth with regard to above- and belowground plant biomass. Since all microcosms showed comparable plant growth and any treatment occurred in the same way, the results of the different environmental inocula should be comparable to each other.

Photosynthetic activity of rice plants is directly driven by the availability of CO_2, as well as that of water, nutrients, light and also indirectly by multiple other factors (Pump and Conrad, 2014). Photosynthetic fixation of $^{13}CO_2$ was similar in all microcosms, reaching stable values of 3.53 ± 0.93 % of the initial concentration after pulse-labeling within 8 h. Since these values were about two orders of magnitude higher compared with the ambient CO_2 concentration (IPCC, 2014) it was assumed that CO_2 fixation and production of the rice plant were in an equilibrium at this concentration. However, previous studies suggested a close link between photosynthesis and rhizospheric processes (Kuzyakov and Gavrichkova, 2010). An effect on the carbon metabolisms of plants by use of a synthetic soil-system instead of normal rice paddy soil was suggested by (Pump and Conrad, 2014).

No differences in the amount of ^{13}C labeling for either aboveground or root plant biomass could be observed with respect to the different inocula of the soil-systems. Even if the ^{13}C values of aboveground plant biomass in the 100 % rice paddy soil control were slightly higher compared to the synthetic soil-systems, a significant difference in ^{13}C accumulation into the plant biomass could not be confirmed by our results **(Figure 3.7)**. It might be possible that the implemented modifications of the soil-systems compensated for this. However, ^{13}C accumulation by translocation of assimilated $^{13}CO_2$ was consistently higher in the aboveground plant biomass than in the plant root for all microcosms. A predominant incorporation of photosynthetically fixed carbon in the aboveground biomass of rice plants was also described before (Kimura et al., 2004; Wu et al., 2009; Pump and Conrad, 2014; Ge et al., 2015). Other studies suggested a connection between the preferential assimilation of $^{13}CO_2$ in the aboveground plant and a higher aboveground plant biomass in total (Pump and Conrad, 2014). This coherence could not be confirmed by our results, since the belowground plant biomass was at the same level or even higher in some of the microcosms. This may be a result of the higher volumes for soil-systems used in this study. However, statistical analysis by ANOVA revealed that neither plant growth parameters nor ^{13}C translocation into the plant biomass could explain differences in bacterial **(Figure 3.13)** and methanogenic community composition **(Figure 3.20)** of the different soil-systems used in this study.

A single pulse of $^{13}CO_2$ with a relatively short incubation time of 5 days was used for labeling in order to ensure that the produced $^{13}CH_4$, as well as the ^{13}C-labeled precursors for the formation of CH_4, were exclusively formed from freshly released rhizodeposits. This could be confirmed, since no stabilization of labeled carbon occurred in humic soil substances of the rhizospheric soil **(Figure 3.7)**. Furthermore, this assumption would also require that the amount of ^{13}C was higher in previous carbon pools and accordingly decreases due to translocation from aboveground- to root plant biomass and further to precursors for the formation of methane and finally to methane. However, it has to be mentioned that the ^{13}C atom % exes values only reflect the relative contribution of plant derived carbon to the respective carbon pool. These relative values are essentially dependent on the pool size. Based on the differences with respect to the different microcosms, pool size of above- and root plant biomass could be assumed to be similar **(Figure 3.3)**. Therefore, higher ^{13}C labeling in the above ground plant biomass indicates reliability of the microcosms. Higher labeling of porewater substances and emitted methane than root biomass in all microcosms (except those based on the mixed inoculum) could therefore be a result of a much higher unlabeled carbon content in the biomass compared to the respective carbon pool. Furthermore, ^{13}C measurement of root samples was only focusing on the root biomass itself rather

than on labeled root released substances. Therefore, photosynthetically fixed ^{13}C allocated to the plant root, which was not converted to plant biomass, but rather used for the plant's metabolisms or directly released as root exudates, may be not detected. Hence, ^{13}C labeling of root exudates might be much higher compared to the root biomass. Furthermore, microcompartments in soil porewater with differences in labeling could not be excluded. Previous pulse-labeling studies with $^{13}CO_2$ and $^{14}CO_2$ revealed that no allocation of labeled CO_2 from the headspace into the soil occurred under flooded conditions without the presence of a plant (Sanaullah et al., 2012; Hernández et al., 2015). Considering all of this it was assumed that the translocation of ^{13}C to porewater substances, as well as labeled methane, were exclusivity originated by release of rhizodeposits.

The labeling pattern of CO_2 dissolved in the porewater may reflect a direct exchange with the CO_2 of the headspace by diffusion through the rice plant, or may have originated from CO_2 production in the rhizosphere. Both processes could not be distinguished completely, but the labeling rates of porewater CO_2 could be considered for contribution of both processes to labeling of CO_2. The ^{13}C enrichment in porewater CO_2 of digested sludge microcosms was at its maximum at 8 h after labeling while decreasing afterwards **(Figure 3.8)**. Since $^{13}CO_2$ in the headspace was absent after 8 h, it might be that the measured $^{13}CO_2$ in the porewater of digested sludge originated from diffusion of $^{13}CO_2$ through the aerenchyma tissue of the rice plants. The same was observed for mixed inocula microcosms, whereas the ^{13}C labeling of porewater CO_2 was stable until 32 h after labeling in this soil-system. This indicates that porewater $^{13}CO_2$ in mixed inocula microcosms may at least not exclusively arise from diffusion of $^{13}CO_2$ from the headspace, but rather also originates from degradation of labeled root released carbon compounds. Since ^{13}C labeling of porewater CO_2 did not reach the maximum before 56 h in the rice paddy soil-system and before 80 h in the 100 % rice paddy soil control, it is thought that $^{13}CO_2$ in these microcosms was primarily formed by the degradation of labeled root released carbon compounds, instead of reaching the rhizosphere by diffusion. Nevertheless, CO_2 which was allocated to the rhizosphere via diffusion through the plants aerenchyma tissue is also assumed as rhizodeposition (Pinton et al., 2001; Singh et al., 2004) and therefore was relevant to this study in itself, as well as other carbon compounds formed by its degradation. Furthermore, $^{13}CO_2$ could also originate from oxidation of $^{13}CH_4$ via methane-oxidizing bacteria. However, an uncertain amount of ^{13}C labeled propionate, acetate, and CH_4 could originate from the conversion of porewater $^{13}CO_2$, since labeling of CO_2 was higher or on the same level as for propionate and CH_4 in the respective microcosms.

Based on the experimental set-up, the microcosms could only be assumed as closed systems during measurement periods, since loss of ^{13}C by $^{13}CH_4$ emission and release of $^{13}CO_2$ in between

could not be determined. Therefore, reliability of microcosms as well as carbon translocation could not be confirmed by mass balance. However, our results for ^{13}C accumulation in aboveground- and root biomass, as well as emitted methane of synthetic rice paddy soil microcosms were similar to those of the original model system (Pump and Conrad, 2014), whose reliability could be confirmed by ^{13}C mass balance (Pump, 2012). In summary, formation of ^{13}C labeled precursors for methane as well as $^{13}CH_4$ was assumed to be exclusively formed by degradation of fresh photosynthates.

However, the contribution of recent plant-assimilated carbon to emission of methane R_m was only about 10 % or lower for microcosms based on the different soil-systems as well as the control. This value is considerably lower compared with steady state labeling approaches, which revealed that up to 60 % of emitted methane is produced from root derived carbon (Yuan et al., 2012). This suggests further that the measured $^{13}CH_4$, as well as its precursors, exclusively originated from freshly released root derived carbon. The same assumption was made in previous single pulse experiments with similar labeling conditions (Pump and Conrad, 2014).

All microcosms contained methanogenic archaea in the rhizospheric soil, as well as on the root surface **(Figure 3.10)**, and furthermore showed a distinct emission of methane **(Figure 3.6)**. Despite the modifications to increase methanogenic activity in rice paddy soil-system based microcosms, the total emission of methane was not significantly higher than in the unmodified plant-soil model system. Rather, the total emission of methane from the rice paddy soil-system of our study (5.15 ± 2.42 nmol g_{dw}^{-1} h^{-1}) was at the same rate as in previous studies without modifications (5.02 ± 1.55 nmol g_{dw}^{-1} h^{-1}) (Pump and Conrad, 2014). It should furthermore be mentioned that the methane emission from the 100 % rice paddy soil control of the previous study (18.87 ± 1.98 nmol g_{dw}^{-1} h^{-1}) was noticeably higher than in our experiments (15.10 ± 1.84 nmol g_{dw}^{-1} h^{-1}). This implies that the effect of implemented modifications could be underestimated, since methanogenic activity also depends on the inoculated rice paddy soil sample itself, which could not be reliably compared to previous studies.

Nevertheless, the presence of methanogenic archaea on the rice root surface of the rice paddy soil-system based microcosms was about 5 times higher with our modifications compared to the unmodified system (Pump et al., 2015). As has been mentioned before, it was possible to show that the approaches employed in this study can be used with environmental samples for inoculation beyond rice paddy soil and result in colonization of the rhizosphere by methanogenic archaea, as well as formation of methane. Furthermore, the total methane emission from

microcosms based on other inocula than rice paddy soil was even higher compared to this based on the rice paddy soil-system.

4.2 Patterns of microbial colonization and carbon translocation in the rhizosphere of different soil-systems

Since rice plants grow in soil, they are placing their roots in direct proximity to a high abundance of microbial diversity (Tringe et al., 2005), making the roots one of the favorable habitats for colonization by soil microorganisms (Kimura et al., 1988). Not only the plant itself but also the soil type shapes the structure and function of microbial communities in the rhizosphere, since the soil represents the main reservoir for rhizosphere microorganisms (Berg and Smalla, 2009). Furthermore, the soil type also has a clear influence on the release of root exudates (Pinton et al., 2001). However, the colonization of rice roots by methanogenic communities is essentially determined by the soil type or, to be more precise, by its function as a seed bank for microbial colonization (Conrad et al., 2008). Although rice was not actually planted in soil types in this study, each soil-system is assumed to fulfill the same functions and was used for reasons explained above. Nevertheless, the question in how far the initial microbial communities of the different soil-systems are of concern for microbial colonization of the rhizosphere remains.

The colonization of the rhizosphere by bacteria and methanogenic archaea **(Figure 3.10)**, as well as the translocation of the recently assimilated plant carbon to the rhizosphere **(Figure 3.7)** produced different results with respect to the different soil-systems and the initial microbial communities they contained. The effect of the soil type on the translocation of recently assimilated carbon has already been described in previous studies (Pump and Conrad, 2014). Since the translocation of carbon to the rhizosphere in the rice paddy soil-system was different from the 100 % rice paddy soil control, an alternating effect of the soil content could be confirmed, as well.

While the carbon compounds released by the rice root serve as available substrates for microbial degradation to methane (Dannenberg and Conrad, 1999; Lu et al., 2005; Pump and Conrad, 2014) this reaction is not performed by a single group of microorganisms but rather by a syntrophic microbial community (Thauer, 1998). Intermediates, like fatty acids, alcohols, acetate, H_2, and CO_2, produced by fermenting bacteria, can be used directly by methanogenic archaea for the conversion to CH_4 (Conrad, 2002; Schink and Stams, 2013). As an alternative, short chain fatty acids, like

propionate originating from the conversion of root released carbon compounds, can be further degraded to acetate, CO_2, and H_2 (Krylova et al., 1997) and consequently provide precursors for the formation of methane (Conrad, 2002; Schink and Stams, 2013). Afterwards, methane may be transported to the atmosphere via the aerenchyma tissue of the rice plant (Minoda and Kimura, 1994; Nouchi et al., 1990) or oxidized by methane-oxidizing bacteria colonizing the rhizosphere (Frenzel, 2000). However, in this study only short chain fatty acids of acetate, propionate, formate, and pyruvate could be detected. Since no enrichment of ^{13}C could be detected in either formate or pyruvate in any of the microcosms, it was assumed that rhizodeposits degraded to formate and pyruvate were rapidly converted further to CO_2 and acetate, or that root derived carbon did not contribute to their formation at all. As has been mentioned before (see 4.1), the translocation of recently assimilated carbon to porewater substances and methane was assumed to have exclusively originated from the release of rhizodeposits by the rice plant.

Microcosms based on rice paddy soil-system showed a lower bacterial colonization of the rhizosphere compared to the other microcosms. Nevertheless, the rhizosphere was colonized by bacteria and their absolute abundance was slightly higher in the rhizospheric soil than in the soil before planting. Although the bacterial colonization in the rice paddy soil-system was low, these microcosms showed by far the highest translocation of root derived carbon to porewater propionate, acetate, and CO_2 **(Figure 3.7)**. In contrast to the other soil-systems, porewater CO_2 in the rice paddy soil-system showed a stable ^{13}C enrichment at its maximum, indicating some availability of rhizodeposits in the preceding carbon pools. The conversion rate of recently root derived carbon to acetate was higher than in the other soil-systems, and the conversion rate to propionate was also high. Based on carbon translocation to the rhizosphere, the rice paddy soil-system is thought to posses a bacterial community composition which was able to degrade root derived carbon more effectively than that of the other microcosms and/or had a higher dependency on rhizodeposits since initial soil organic carbon in this soil-system was the lowest **(Table 3.2)**.

Whereas colonization by methanogenic archaea in the rice paddy soil-system seemed to take place mostly on the root surface, colonization by methane-oxidizing bacteria primarily took place in the rhizospheric soil. The absolute abundance of methanogens in the rhizospheric soil was lower compared to the other microcosms, while that on the root surface was at the same level as in the other soil-systems. According to the low number of methanogens in the soil before planting, the methanogenic community of the rice paddy soil-system either seems to be quite capable of colonizing the root surface, or had a higher need of spatial proximity to root derived carbon, due to

the low amount of organic carbon in the initial soil-system. However, in the rice paddy soil-system, both the absolute abundance of methanogenic archaea in the entire rhizosphere and the total emission rate of methane **(Figure 3.6)** were low. In contrast to this, the enrichment of ^{13}C of methane **(Figure 3.7)**, as well as the contribution of carbon recently assimilated by the plant to the total methane emission **(Figure 3.9)** was by far the highest in the rice paddy soil-system. Therefore, the methanogenic community composition of this soil-system is thought to use intermediates originating from the degradation of root derived carbon more effectively and/or to have a higher dependency for rhizodeposits because of the low amount of initial soil organic carbon in this system. Because of the relation of the rice plant and the inoculated rice paddy soil, it might be possible that the bacterial as well as the methanogenic community of the rice paddy soil-system were better adapted for the degradation of rice root derived carbon to methane. However, the emission of methane originating from root derived carbon was also high, but just as high as in the case of the digested sludge soil system.

For microcosms based on the mixed inoculum soil-system, bacterial colonization of the rhizospheric soil was lower compared to the soil before planting and was in general as low as in the rice paddy soil-system. Therefore, the bacterial community of the mixed inoculum soil-system had either a low capability or a low necessity for colonization of the rhizospheric soil. In contrast to this, the bacterial colonization of the root surface was high. However, as opposed to the rice paddy soil-system, microbial translocation of root derived carbon to propionate, acetate and CO_2 was much lower in microcosms based on the mixed soil-system. Conversion rates of rhizodeposits to propionate were also lower than in the other microcosms. Considering this, the initial soil organic carbon seems to be a more important source for bacterial degradation of organic matter in the mixed inoculum soil-system than rhizodeposits. Therefore, the rhizosphere of the mixed inoculum soil-systems is thought to posses a bacterial community composition which is able to degrade the initial soil organic carbon more effectively and/or to have almost no need for rhizodeposits due to the high amount of soil organic carbon in this system. However, the mixed inoculum also possessed a contingent of inoculated rice paddy soil. Therefore, if the above assumption is correct and bacterial groups in the rice paddy soil were better adapted to the rice root, these groups may be absent or of low abundance in the rhizosphere of the mixed soil-system, or they did not contribute to the conversion of root derived carbon at all due to the higher availability of initial soil organic carbon.

The colonization by methanogenic archaea in the mixed soil-system was similar for rhizospheric soil, root surface, and soil before planting, which indicates no considerable preference of colonizing the rhizosphere. Considering this as well as the fact that the bacteria seemed to feed more on initial soil organic carbon rather than on rhizodeposits, intermediates of degradation usable for methanogenesis might not be more available in the rhizosphere compared to the rest of the soil. Nevertheless, absolute abundance of methanogens on the root surface was at the same level as in the rice paddy soil-system and even slightly higher in the rhizospheric soil, which would in turn be compatible with the higher total CH_4 emission from the mixed soil-system. The enrichment of ^{13}C within methane as well as the contribution of recently plant-assimilated carbon to the total methane emission were much lower than in the rice paddy soil-system, but on the same level as the digested sludge soil-system. The emission of methane originating from root derived carbon was also lower than in the other microcosms. Therefore, the methanogenic community of the mixed soil-system is thought to use intermediates originating from the degradation of initial soil organic carbon more effectively and/or to have a lower dependency on rhizodeposits due to the high amount of initial soil organic carbon in this system. Furthermore, methane-oxidizing bacteria showed a preference for the colonization of the rhizospheric soil rather than for the other soil compartments. Since the mixed inoculum was a combination of rice paddy soil and digested sludge, colonization of the rhizosphere could be assumed to be an average between the rice paddy soil-system and the digested sludge soil-system. However, it has to be mentioned that the determination of microbial abundance in the soil before planting took place at a different point in time then for rhizospheric soil and the root surface. Hence, the abundance of microorganisms capable of colonizing the rhizosphere might have developed differently between these two points in time. Therefore, some microorganisms might not have been available for colonizing the rhizosphere. Furthermore, it could be assumed that the same microorganisms which were colonizing the rhizospheric soil in the rice paddy soil-system were less dependent on doing so in the mixed inoculum because of the higher amount of initial soil organic carbon. The abundance of the microorganisms colonizing the rhizospheric soil of the digested sludge contingent was, however, decreased due to a dilution caused by the mixture of these two inocula.

The absolute abundance of bacteria in the rhizospheric soil of microcosms based on the digested sludge soil-system was higher compared to the other soil-systems. Although the absolute abundance of bacteria was the highest of all microcosms in the entire rhizosphere, the number of bacteria was high even before planting and therefore no differences with regard to colonization could be observed for colonization between rhizospheric soil and soil before planting. Despite the

high number of bacteria in the entire rhizosphere, microbial degradation of rhizodeposits to propionate and acetate was in between that of the rice paddy soil-system and that of the mixed inoculum soil-system. The contribution of root derived carbon to the formation of acetate and propionate was higher in the digested sludge than in the mixed inocula microcosms, but lower than in those based on the rice paddy soil-system. Therefore, the rhizosphere of the digested sludge soil-system was thought to posses a bacterial community composition which is able to degrade root derived carbon but not as effectively as in the rice paddy soil-system, despite also having the highest amount of initial soil organic carbon.

The colonization of the rhizospheric soil by methanogenic archaea was far higher in the digested sludge soil-system compared to the other microcosms, while the absolute methanogenic abundance on the root surface was the same as in the other soil-systems. Furthermore, methane-oxidizing bacteria also preferred the rhizospheric soil of the digested sludge microcosms for colonization, and their abundance on the root surface was far higher than in the other microcosms and in the soil before planting. In the microcosms based on the digested sludge system, methanogenic archaea clearly preferred colonizing the rhizospheric soil, since their presence on the root surface was even lower than in the soil before planting. In accordance to the highest absolute abundance of methanogens in the entire rhizosphere, the total methane emission from these microcosms was also far higher.

Despite the numerous occurrence of methanogens in the rhizosphere, ^{13}C enrichment within methane, as well as the contribution of recently derived plant carbon to the total emission of methane, was as low as in the mixed inoculum soil-system. Nevertheless, emission of methane originating from root derived carbon was as high as in the rice paddy soil-system. Therefore, the rhizosphere of the digested sludge soil-system was thought to posses a bacterial and methanogenic community composition which is able to degrade root derived carbon to methane, but not as effectively as in the rice paddy soil-system. Furthermore, rhizodeposits play a role in the formation of methane in microcosms based on the digested sludge soil-system, despite the fact that the digested sludge soil-system had the highest amount of initial soil organic carbon.

Considering all of this, the potential of the rhizospheric bacterial community to degrade root derived carbon to precursors for formation of methane could not just be explained by the absolute abundance of rhizospheric bacteria, nor by the amount of initial soil organic carbon of the different soil-systems. The same holds true for the absolute abundance of methanogenic archaea and the formation of methane from root derived carbon. Nevertheless, microcosms with a high emission

rate of total methane also possessed a high number of methanogenic archaea in the rhizosphere, indicating a link between colonization of the rhizosphere by methanogenic archaea and the formation of methane. These outcomes were contrary to the results of (Pump et al., 2015), which showed the abundance of methanogenic archaea to increase linearly with the emission of methane originating from root derived carbon, but rates of total methane could not be systematically related to the number of methanogens. It has to be mentioned that a correlation between the abundance of methanogenic archaea and the emission of methane originating from root derived carbon of those previous study could only be determined until the vegetative growth stage. Since the rice plants of our study were considered to be at the reproductive stage, the turning point of this correlation may already have been exceeded. Furthermore, the relation between the total emission of methane and the number of methanogenic archaea was investigated with regard to the methanogenic abundance of the soil plus the roots, whereas that of the rhizosphere was considered in our study. However, since the translocation of recently assimilated plant carbon to the rhizosphere is affected by the microbial soil community (Pump and Conrad, 2014), it is thought that the degradation of root derived to methane depends more on the bacterial and methanogenic community composition and not merely on the absolute abundance in the rhizosphere or on the amount of initial soil organic carbon.

Furthermore, in almost none of the different rhizospheric compartments of all the microcosms, the absolute abundance of bacteria, methanogenic archaea, or methane-oxidizing bacteria was higher with respect to the other microcosms, if not already also in the soil before planting. Therefore, microbial colonization of the rhizosphere by bacteria and methanogenic archaea is thought to depend on the absolute abundance of the initial soil microbial communities of the different soil-systems.

4.3 Impact of the bacterial community composition on the degradation of root derived carbon to methane in the rice rhizosphere

Although interactions between rice plants and the microbial community of the rhizosphere are substantially more versatile, this study focused on the interactions based on the release of rhizodeposits by the plant. The translocation of carbon, which was fixed by the rice plant via photosynthesis, was different with respect to the investigated microcosms **(Figure 3.7 and 3.8)**. Previous studies showed that this translocation is affected by the microbial soil community (Pump and Conrad, 2014). Our results indicated a link between the amount of ^{13}C-labeling of porewater

CO_2, acetate, the methane emission rate originating from recently derived root carbon, and the bacterial community composition of the rhizosphere **(Figure 3.13)** rather than the absolute bacterial abundance.

The composition of the bacterial community in the different microcosms was investigated by illumina sequencing analysis. The number of reads and the number of the observed OTUs made a distinction between the tested soil-systems, but not between the different soil-compartments **(Table 3.3)**. Nevertheless, we obtained at least 1,500 high-quality sequence reads for each compartment and soil-system. Despite the fact that OTU- and read number, as well as species richness (Chao1 estimator) of microcosms based on digested sludge were lower compared to the other systems, the predicted coverage (Good's coverage) and diversity (Shannon index) were only slightly lower than in the other microcosms. Even if a higher number of reads would result in a further increase of the samples coverage in the digested soil-system, at least 89 % of the bacterial community composition was revealed according to predicted coverage **(Figure 3.11)**. Therefore we assume to have covered the essential part of the bacterial community structure in order to assure reliable results. Nevertheless, bacterial diversity in the rhizosphere was lower in digested sludge compared with the other microcosms. OTU-level analysis of the diversity evenness (Pielou`s evenness) revealed also that the bacterial community composition of the microcosms based on digested sludge was different compared to the other microcosms, but that it showed great similarities at the phylum-level with that of other anaerobic digesters from previous studies (Sundberg et al., 2013). However, a comparison of the bacterial abundance in the digested sludge soil-system with that of regular digested sludge have to be done with caution, since most other studies used recently incubated sludge, while in this study the digested sludge was dried and inoculated in an inert soil-matrix, which might affected the composition of the bacterial community drastically.

Except in the digested sludge soil-system, the rhizospheric soil and the root surface was colonized by the bacterial phyla of Proteobacteria, Firmicutes, Actinobacteria, Gemmatimonadetes, Chloroflexi, Deinococcus-Thermus, Bacteroidetes, Acidobacteria, and Planctomycetes **(Figure 3.12)**. Since the digested sludge samples had the lowest diversity at OTU-level, a lack of diversity at the phylum-level could also be a result of this. However, changes in the bacterial community composition of the rice-planted microcosms with respect to the different soil compartments revealed that the rice rhizosphere is a distinct habitat for bacterial colonization. Before the planting of the rice plants, Proteobacteria and Firmicutes were the most abundant phyla within the bacterial

communities of all microcosms. The same was shown in other studies focusing on bacterial colonization of rice roots in plant-soil microcosms based on rice paddy soil (Lu et al., 2006; Hernández et al., 2015). Except for that based on digested sludge, the rhizospheric soil of all microcosms showed a higher colonization by Proteobacteria than the soil before planting, while the abundance of Firmicutes in the rhizospheric soil was lower than in the unplanted soil. The presence of Proteobacteria directly on the root surface was even higher, while that of Firmicutes was actually lower compared to the unplanted and rhizospheric soil. The same differences between rhizospheric soil and root surface with regard to the colonization by Firmicutes (Hernández et al., 2015) and Proteobacteria (Edwards et al., 2015; Hernández et al., 2015) were observed before. Such differences between rhizospheric soil and root surface may reflect the different physio-chemical characteristics in these compartments.

The bacterial colonization of the rhizosphere in microcosms based on digested sludge was also different from that in the soil before planting, but here the phylum of Bacteroides was dominant and likewise highly abundant in the rhizosphere. This stood in contrast to the other microcosms as well as to previous studies, which also focused on bacterial colonization of rice roots in plant-soil microcosms based on rice paddy soil, but in which the phylum of Bacteroides only plays a minor role (Hernández et al., 2015). As was the case in the other microcosms, the Proteobacteria represented the dominant phylum on the root surface also in those microcosms based on digested sludge. Furthermore, the abundance of Firmicutes was also lower in the rhizospheric soil compared to the soil before planting, and even lower on the root surface, as was the case in the other microcosms.

4.3.1 Link between bacterial groups and the degradation of root derived carbon

Considering several biotic and abiotic parameters, it could be shown that neither plant factors like plant height and plant biomass, nor organic carbon content of the different soil- systems, had an effect on the bacterial community structure of the rice rhizosphere. The amount of ^{13}C-labeling for CO_2 and acetate, as well as the $^{13}CH_4$ emission rate showed a correlation with the rhizospheric community composition **(Figure 3.13)**. Several OTUs, whose abundance was linked to the emission of methane originating from recently assimilated root derived carbon, could be identified **(Figure 3.14)**.

OTU_3 and OTU_4 could be assigned to the genus of *Anaeromyxobacter* and could be linked to the formation of methane from rhizodeposits. The genus of *Anaeromyxobacter* is described as dissimilatory iron reducing bacteria, which are able to oxidize acetate (Lovley et al., 2004), as a central intermediate in anaerobic degradation of organic matter (Hori et al., 2007), while some strains are also able to reduce nitrate (Treude et al., 2003). Species of *Anaeromyxobacter* have previously been identified to colonize the rhizosphere of rice (Treude et al., 2003; Scheid et al. 2004; Edwards et al., 2015). OTU_3 showed intense colonization of the rhizospheric soil in microcosms based on mixed inoculum, on the root surface of the 100 % soil control, as well as in the entire rhizosphere of the rice paddy soil-system microcosms. OTU_4 only showed a high abundance on the root surface in the rice paddy soil-system. In all microcosms except those based on digested sludge, OTU_3 and OTU_4 were more abundant in the rhizospheric soil and/or on the root surface than in the unplanted soil, which indicates a tendency of *Anaeromyxobacter* to colonize the rice rhizosphere. This could be a result of the re-oxidation of Fe(II) to Fe(III), and maybe also of re-oxidation of nitrite to nitrate. The reason for this may be the oxic influence of the rice root (Brune et al., 2000; Liesack et al., 2000; Conrad and Frenzel, 2002), since Fe(III)-oxides, ready for direct utilization might have a poor availability in rice field soil, e.g. be available in crystalline rather than amorphous iron oxide phases (Roden, 2003; Roden, 2006). Furthermore, OTU_4 showed a preference for the colonization of the root surface rather than for the rhizospheric soil. In general, *Anaeromyxobacter* seemed to slightly prefer the root surface for colonization instead of the rhizospheric soil, at least in the rice paddy soil-system as well as in the control. Therefore, the availability of Fe(III) and nitrate might be higher on the root surface compared to the rhizospheric soil, since a direct proximity to the root may increase the presence of oxygen.

However, *Anaeromyxobacter* colonizing the rice roots are known to be able to assimilate acetate under methanogenic conditions, i.e. when CO_2 is the predominant electron acceptor (Hori et al., 2007). This may imply that methanogenesis in the rhizosphere was not totally suppressed by the presence of reducible electron acceptors such as Fe(III) and nitrate, but as the microbial reduction of Fe(III) and nitrate by *Anaeromyxobacter* was coupled to the oxidation of acetate, this is thought to be the thermodynamically favorable process instead of acetoclastic methanogenesis. Nevertheless, acetoclastic methanogenesis is also likely to have occurred in the rhizosphere since the availability of Fe(III) and nitrate are coupled to the release of oxygen, which is known to be a dynamic phenomenon in the rhizosphere (Flessa and Fischer, 1992). However, considering this together with the fact that the abundance of *Anaeromyxobacter* in the rhizosphere was linked to the emission of $^{13}CH_4$, this could further imply that H_2/CO_2 might be a more important source for the formation of methane from root derived carbon on the root surface. This, then, could indicate a

preference for hydrogenotrophic instead of acetoclastic methanogenesis on the root surface in the rice paddy soil-system and in the control, since acetate assimilation by *Anaeromyxobacter* might indicate CO_2 as the predominant electron acceptor for methanogenesis (Hori et al., 2007). Hydrogenotrophic methanogenesis was thought to be the main pathway for the formation of methane on rice roots before (Conrad, 2007). However, for the mixed inoculum soil-system this assumption does not count because the colonization by *Anaeromyxobacter* was almost the same in both rhizospheric soil and root surface. Furthermore, OTU_3 and OTU_4 were totally absent in the entire rhizosphere of the digested sludge microcosms, and also from the soil before planting.

OTU_8 (*Bradyrhizobium*) was also linked to the emission of $^{13}CH_4$ and furthermore showed a preference for the colonization of the root surface, but only in the rice paddy soil-system. In microcosms based on the mixed inoculum, OTU_8 was just slightly more frequent in the rhizospheric soil and on the root surface than in the soil before planting. The genus of *Bradyrhizobium* is characterized as nitrogen-fixing plant symbiotic bacteria (Kaneko et al., 2002) and has previously been described for colonizing rice roots (Tan et al., 2001).

Inoculation of rice roots with strains of *Bradyrhizobium* resulted in an increased uptake of N, P and K by the plants (Biswas et al., 2000). Therefore the colonization of rice roots with *Bradyrhizobium* might in some way benefit the release of root derived carbon compounds, which can serve as precursors for $^{13}CH_4$. This is plausible since the contribution of plant derived carbon to porewater propionate, acetate, and CO_2 was higher in the rice paddy soil-system compared to the other soil-systems.

OTU_5 (*Kineosporiaceae*) within the Actinobacteria showed a high abundance on the root surface in the rice paddy soil-system and could be linked to the formation of methane from root derived carbon. Furthermore, OTU_5 was also present in the entire rhizosphere of the control. However, the colonization of the root surface was higher compared to the rhizospheric soil of the rice paddy soil-system as well as the control, which seems plausible since *Kineosporiaceae* have been described as aerobic bacteria (Trujillo, 2001). *Kineosporiaceae* have previously been reported in environments like soils, plant tissues and aerobic sludge, and have also been reported to colonize the rice rhizosphere (Edwards et al., 2015).

Kineosporiaceae are known to degrade glucose and cellobiose under oxic conditions (Schellenberger et al. 2010), which could explain their link to $^{13}CH_4$ emission due to their function in

the degradation of rhizodeposits to precursors for the emission of methane, as well as their predominant presence on the root surface due to the proximity to the root exudates. A previous study also suggested that the bacterial community directly colonizing the surface of rice roots is more involved in the fermentation of root derived carbon compared to that of the rhizospheric soil (Hernández et al., 2015). *Kineosporiaceae* seem to have the highest abundance in the rice paddy soil-system compared with the other microcosms, which seems plausible to their link to the contribution of porewater propionate, acetate, and CO_2, by the degradation of rhizodeposits, which was also the highest in the rice paddy soil-system. Further members of the class of Actinobacteria could also be found in the rhizospheric soil and on the root surface of rice plants in previous studies (Edwards et al., 2015; Hernández et al., 2015). Like OTU_5, the Actinobacteria in these studies also preferred to colonize the root surface rather than the rhizospheric soil, which indicates preference for the proximity to rhizodeposits, as well as to root released oxygen.

OTU_160 (*Bacteroidales*, BA008) colonized the entire rhizosphere of the digested sludge microcosms and could also be linked to the emission of $^{13}CH_4$. In all other microcosms OTU_160 was absent from the rhizosphere, as well as from the soil before planting. In the digested sludge microcosms, OTU_160 was frequent in the rhizospheric soil and even more abundant on the root surface. Members of the order of *Bacteroidales* are thought to represent key populations in a range of anoxic/anaerobic environments (Tourlousse et al., 2015), which are rich in organic carbon (Caumette et al., 2015). These conditions are thought to be available in the rhizosphere of microcosms based on the digested sludge soil-system. Strain 6E of *Bacteroidales* could be isolated from Japanese rice paddy soil (Tourlousse et al., 2015), while strain TBC1 belongs to a hitherto uncultured cluster within the *Bacteroidales*, named BA008, which was isolated from a methanogenic wastewater treatment system (Tourlousse et al., 2015). Since OTU_160 could not be detected in the soil before planting of the mixed inoculum, the *Bacteroidales* contained therein are thought to have originated from the digested sludge component in this inoculum and were therefore not be able to deal with the conditions caused by the rice paddy soil component, not even in the first days of flooding without the presence of the rice plant.

However, strains of *Bacteroidales* are described as strictly anaerobe bacteria, which are capable of fermenting various carbohydrates. In accordance with known root exudates of rice plants (Lin and You, 1989; Aulakh et al, 2001), *Bacteroidales* can degrade glucose, xylose, and cellobiose via fermentation (Tourlousse et al., 2015). Therefore, the degradation of such root derived carbon sources are thought to be a link to the emission of $^{13}CH_4$. Since the colonization of *Bacteroidales*

occurred predominantly on the root surface, a strictly anaerobic way of life could not be assumed for OTU_160. Nevertheless, since *Bacteroidales* are assumed to be involved in the degradation of some root released carbon compounds, the preference for colonizing the root surface would correspond to previous studies, in which bacteria directly colonizing the surface of rice roots have been suggested to be more involved in the fermentation of root derived carbon than that of the rhizospheric soil (Hernández et al., 2015). Furthermore, members of the phylum of Bacteroidetes could also be found in the rhizospheric soil and on the root surface of rice plants in the previous study. Like for OTU_160 the Bacteroidetes in this study showed a preference for colonizing the root surface compared to the rhizospheric soil. Even if the contribution of plant derived carbon to propionate, acetate, and CO_2 in microcosms based on digested sludge was not as high as in the rice paddy-soil system it was higher than in the mixed inoculum-system and therefore rhizodeposits are considered to play a role for bacterial carbon degradation processes. Considering this as well as the high abundance of OTU_160 in the rhizosphere of the digested sludge soil-system, *Bacteroidales* are assumed to be primarily responsible for the degradation of root derived carbon to precursors for the formation of methane in the digested sludge microcosms.

Further OTUs, which are linked to $^{13}CH_4$ emission belonged to the phyla of Firmicutes, Gemmatimonadetes, Fibrobacteres, Cyanobacteria, and Chloroflexi. However, all these OTUs showed either no considerable colonization of the rhizospheric soil and/or the root surface in comparison to the soil before planting, or their abundance was very low, like OTU_29 (*Desulfosporosinus*) and OTU_41 (*Pelotomaculum*).

Previous studies identified *Azospirillum* (Lu et al., 2006; Hernández et al., 2015) and members of the family of *Burkholderiaceae* (Lu et al., 2006) as some major consumers of rice root released carbon. Although no OTUs of *Azospirillum* could be linked to $^{13}CH_4$ emission in this study, some *Azospirillum* related OTUs which are directly linked to the formation of $^{13}CO_2$ or ^{13}C-labeled acetate, might be revealed by further analysis. Nevertheless, OTU_27 and OTU_57 could be linked to $^{13}CH_4$ emission. They also colonized the root surface of microcosms based on the rice paddy soil-system, and belong to the order of *Burkholderiales,* even if they were not classified as *Burkholderiaceae* but as *Comamonadaceae*. Previous studies of (Lu et al., 2006) were based on terminal length polymorphism analysis (t-RFLP) and clone libraries, which have a lower resolution and sensitivity than deep sequencing. Since (Hernández et al., 2015) also used deep sequencing (even though 454-pyro sequencing was used in-stead of illumina sequencing) a lower resolution

and sensitivity should not matter too much. However, comparisons of the results have to be done with caution, since previous studies were not focusing on the degradation of recently assimilated plant derived carbon, but also on root derived carbon which might have been translocated to some intermediates of soil organic carbon and/or might have been assimilated as microbial biomass and then subsequently consumed by rhizospheric microorganisms. Furthermore, previous studies were focusing on the labeling of bacterial rRNA in order to evaluate the active bacterial community, which consumes the root derived carbon, while our approach was focusing on the bacterial colonization by targeting bacterial DNA for sequencing.

However, OTUs linked to the formation of methane from rhizodeposits in this study do not necessarily feed on rhizodeposits, but can also affect the formation of $^{13}CH_4$ in some other way. Previous studies showed a strong increase in the release of root derived carbon into the rhizosphere due to the presence of rhizospheric microorganisms. This occurs due to syntheses of enzymes or metabolites that can alter the integrity of root cells or the permeability of their membrane. Therefore, root morphology can be modified directly by phytohormones produced by microorganisms in the rhizosphere or indirectly by a change in the availability of nutrients as a result of microbial processes (Nguyen, 2003). Furthermore, the plant health affects the microbial community associated with the roots (Siciliano et al., 2001) and is in return influenced by microorganisms of the rhizosphere (Berg and Smalla, 2009).

In summary, the contribution of functional bacterial groups to the formation of methane from root derived carbon is thought to be versatile. While some genera like *Bradyrhizobium* seem to have a positive effect on the release of degradable carbon compounds by the root, others like *Kineosporiaceae* and *Bacteroidales* seem to be actively involved in the degradation of rhizodeposits to precursors for methane formation. On the root surface of microcosms based on the rice paddy soil-system the genus of *Bradyrhizobium* is thought to affect the release of rhizodeposits and therefore contributes to the high translocation of root derived carbon to methane. Furthermore, representatives of the *Kineosporiaceae*, also primarily colonizing the root surface, might benefit from this and/or effectively degrade the root released carbon compounds of the rice paddy soil-system. In microcosms based on the digested sludge soil-system on the other hand, *Bacteroidales* seemed to be the dominant genus in the entire rhizosphere responsible for the degradation of rhizodeposits. Neither, *Bradyrhizobium*, nor *Kineosporiaceae* nor *Bacteroidales* showed any considerable colonization of the rhizosphere in microcosms based on the mixed

inoculum soil-system, which would also explain the low translocation of root derived carbon to methane in this system.

4.3.2 The rhizosphere of rice as a distinct habitat for bacterial colonization

Because of the influence of the rice root, the rhizosphere represents a distinct soil compartment (Liesack et al., 2000; Conrad, 2007), with specific physical, biological, and chemical conditions (Barea et al., 2005; Manoharachary and Mukerji, 2006). Since the rhizosphere can be further divided into the sections of rhizospheric soil and root surface (Lynch and Whipps, 1990), the respective colonization of each part of the rhizosphere was brought into focus. However, the unique conditions for microorganisms in the rhizosphere bring up the two questions, namely which bacteria show a preference for the colonization of the rhizosphere in all of the microcosms and how is the colonization linked to the degradation of rhizodeposits to methane.

The bacterial OTUs which are linked to $^{13}CH_4$ emission and are ubiquitous in the rhizospheric soil and/or on the root surface of all microcosms, would give a lead about: (i) whether rice plants shape their distinct bacterial community in the rhizosphere; (ii) how this distinct community would be structured; and (iii) how the community composition contributes to the formation of methane from root derived carbon. Since digested sludge showed a unique composition of the bacterial rhizospheric community which is linked to $^{13}CH_4$ emission, no ubiquitous bacterial OTUs, which which were present in the entire rhizosphere of all microcosms could be detected. Nevertheless, OTU_3 (*Anaeromyxobacter*), OTU_4 (*Anaeromyxobacter*), OTU_97 (*Anaeromyxobacter*), OTU_8 (*Bradyrhizobium*), OTU_33 (*Clostridium*), and OTU_5 (*Kineosporiaceae*) were ubiquitous in the rhizospheric soil and on the root surface in the rice paddy soil-system and in the mixed inocula microcosms, as well as in the 100 % soil control. Of all of these OTUs, only OTU_33 was present in the digested sludge soil-system, but only before planting. Since OTU_33 also showed no considerable colonization of the rhizosphere in other soil-systems, a lack of potential or of need for colonizing the rhizosphere may have been prevalent in the digested sludge soil-system. Considering all of this, rice plants might posses the ability to shape a distinct bacterial community structure in their rhizosphere, but the community composition also depends of the initial pool of microorganisms available for colonization. This could also be confirmed by phylogenetic patterns in the bacterial community structure **(Figure 3.13)**. Detrended correspondence analysis (DCA) revealed that, at least in the mixed inoculum and rice paddy soil-system, as well as in the control, the bacterial community composition on the rice root surface was more related to each other than to the soil before planting.

Furthermore, the abundance of the bacteria in the rice-plant microcosms changed with respect to the different soil compartments **(Figure 3.16)**. Therefore, bacterial OTUs were distributed differently across the soil before planting, the rhizospheric soil, and the root surface. This indicates that in all microcosms the rice rhizosphere was a distinct habitat for bacterial colonization and that different OTUs show a preference for colonizing the rhizospheric soil and/or the root surface.

OTU_1 (*Rickettsiales*), OTU_4 (*Anaeromyxobacter*), OTU_5 (*Kineosporiaceae*), OTU_8 (*Bradyrhizobium*), OTU_10 (*Pleomorphomonas*), and OTU_12 (*Methylosinus*) were characteristic for colonizing the surface of the rice roots, while *Rickettsiales* were most abundant in general. Since OTU_1 could not be taxonomically assigned beyond order-level considerations of ecological functions are limited. Nevertheless, the order of *Rickettsiales* has been described to colonize rice roots before (Qiu et al., 2009). In the previous experiments, rice roots were incubated with $^{13}CH_4$ in order to follow the pathway of methane oxidation. Even though the order of *Rickettsiales* was present on the rice roots, only a weak incorporation of ^{13}C into the DNA could be determined, indicating no oxidation of CH_4 as well as no further consumption of CO_2 formed by methane oxidation. However, further declarations regarding a preference of *Rickettsiales* to colonize the root surface could not be made.

Since *Anaeromyxobacter* are described as iron- (Lovley et al., 2004) or nitrate reducing bacteria (Treude et al., 2003), which are able to oxidize acetate originating from the degradation of organic matter (Hori et al., 2007), a preference for colonizing the root surface seems to be plausible because of the availability of nitrate, Fe(III) (Brune et al., 2000; Liesack et al., 2000; Conrad and Frenzel, 2002) and degradable organic matter (Dannenberg and Conrad, 1999; Lu et al., 2005; Pump and Conrad, 2014). This also applies for *Kineosporiaceae*, which are thought to be aerobic bacteria (Trujillo, 2001), able to degrade glucose and cellobiose under oxic conditions (Schellenberger et al., 2010), which may be released as rhizodeposits by the root.

The genus of *Methylosinus* belongs to the type II methanotrophs and was already identified in rice paddy soils as well as for colonizing rice roots (Bodelier et al., 2000; Horz et al., 2001). As the rice rhizosphere is supplied with methane as well as oxygen, the rhizosphere is an ecological niche for methane-oxidizing bacteria, which can be metabolically active in this area of the soil (Gilbert and Frenzel, 1998). Indeed, the abundance of methanotrophs is usually higher on the rice roots

compared to the bulk soil (Conrad, 2007). Therefore, a preference of *Methylosinus* to colonize the root surface is very reasonable. Since the ecological function of the methane-oxidizing bacteria is linked with the conversion of methane rather than the degradation of rhizodeposits, the methanotrophs in general are discussed elsewhere (see 4.2). Nevertheless, CO_2 originating from the oxidation of methane can again serve as a precursor for hydrogenotrophic methanogenesis (Dannenberg and Conrad, 1999). Therefore, methanotrophic bacteria may also contribute to the formation of methane from root derived carbon compounds by oxidizing ^{13}C-labeled methane and thereby providing $^{13}CO_2$ for hydrogenotrophic methanogenesis.

Pleomorphomonas belong to the order of Rhizobiales. Strains of this genus were previously isolated from rice paddy soil (Xie and Yokota, 2005b), as well as from the roots of rice plants (Bao et al., 2014) and *Jatropha curcas* (Jatropha) (Madhaiyan et al., 2013). *Pleomorphomonas* are described as nitrogen-fixing bacteria (Madhaiyan et al., 2013; Xie and Yokota, 2005b), which can also reduce nitrate and utilize different carbon sources (Xie and Yokota, 2005b). In accordance with known root exudates of rice plants (Lin and You, 1989; Aulakh et al., 2001), *Pleomorphomonas* can utilize acetate, pyruvate, D-glucose, D-fructose, sucrose, D-xylose and cellobiose as their carbon sources (Xie and Yokota, 2005b). Despite their potential to degrade some of the root derived carbon compounds, the abundance of OTUs belonging to the genus of *Pleomorphomonas* could not be linked to the emission of $^{13}CH_4$. A reason for this may be that no considerable differences in the abundance of *Pleomorphomonas* in the rhizospheric soil and on the root surface could be determined between the different microcosms, despite these based on digested sludge where *Pleomorphomonas* were absent (data not shown). According to analysis of 16S rRNA, some strains of *Pleomorphomonas* are closely related to the genus of *Methylosinus* (93 % similarity), but they show no potential to oxidize methane (Xie and Yokota, 2005b). Furthermore, methane-oxidizing bacteria and N-fixing *Pleomorphomonas* share a common symbiosis pathway with rice plants. Ca^{2+}/calmodulin-dependent protein kinase is a component of the common mutualistic symbiosis pathway between rice plants and N-fixing bacteria. A lack of Ca^{2+}/calmodulin-dependent protein kinase, caused by a knock-out of the coding gene *OsCCAMK* in wild type rice plants, leads to a lower relative abundance of methane-oxidizing-bacteria as well as N-fixing *Pleomorphomonas*, and therefore somehow links CH_4 flux and N-fixation (Bao et al., 2014). However, the ability to utilize carbon compounds which might be released by the rice root is thought to be the key factor for a preference of *Pleomorphomonas* to colonize the root surface.

OTU_19 (*Azospirillum*) was characteristic for the rhizospheric soil, but with a low abundance. Species of the *Azospirillum* are known to fix nitrogen (Khammas et al. 1989; Xie and Yokota, 2005a) and reduce nitrate (Xie and Yokota, 2005a). *Azospirillum* has been previously described as being associated with rice roots (Khammas et al., 1989; Xie and Yokota, 2005a; Hernández et al., 2015). Furthermore *Azospirillum* was previously mentioned as a major consumer of carbon released by the rice root (Lu et al., 2006; Hernández et al., 2015). *Azospirillum* species can utilize sucrose and D-xylose, which might be released as root exudates (Lin and You, 1989; Aulakh et al., 2001). Furthermore, a positive effect on the growth and development of rice roots is suggested when roots are incubated with *Azospirillum* (El-Khawas and Adachi, 1999). However, the availability of nitrate (Brune et al., 2000; Liesack et al., 2000; Conrad and Frenzel, 2002) in the rhizosphere as well as the ability to utilize sucrose and xylose which might be released by the rice root are thought to be the reason for a preferred colonization of the rhizospheric soil rather than the unplanted soil.

OTU_3 (*Anaeromyxobacter*) and OTU_14 (*Myxococcales*) were characteristic for the rhizosphere in general and a preference for colonization could not be distinguished with regard to the rhizospheric soil and root surface. The same reasons for which *Anaeromyxobacter* (OTU_4) prefer to colonize the root surface (see above) might apply to the colonization of the entire rhizosphere by OTU_3. *Myxococcales* are a diverse order within the Deltaproteobacteria. Taxonomy of OTU_14 could not be assigned beyond order-level and therefore any considerations of their ecological functions are limited. Since the genus of *Anaeromyxobacter* also belongs to the order of *Myxococcales*, OTU_14 might be related to *Anaeromyxobacter*. Related properties might furthermore be an explanation for similarities in the pattern of colonization.

Representatives of *Clostridiaceae* (OTU_2, OTU_15, OTU_16, and OTU_33) were particularly strongly associated with the rhizospheric soil and/or soil before planting, rather than with the root surface. Only OTU_22 and OTU_20 were consistently present throughout all soil compartments and are also classified as *Clostridiaceae*. However, Clostridia are described to colonize the rice rhizosphere (Scheid et al., 2004; Sun et al., 2008) and some of them might be able to fix nitrogen (Sun et al., 2008). The Clostridia colonizing the rice rhizosphere are thought to live on fermentation and are responsible for the production of hydrogen, butyrate, propionate and acetate (Scheid et al., 2004). Nevertheless, most Clostridia isolated from the rice rhizosphere showed to be aerobic and therefore the OTUs belonging to the Clostridia might be other representatives in the present study,

since an anaerobic life style would suggest a colonization of the root surface and/or the rhizospheric soil instead. However, since Clostridia are thought to be sensitive to nitrate (Scheid et al., 2004), re-oxidation of nitrite to nitrate in the rhizosphere might be the reason for their tendency to colonize the unplanted soil. Through the oxic influence of the root this re-oxidation might be even more important on the root surface and may therefore be the reason for a preference for colonization of the rhizospheric soil rather than the root surface.

4.4 Influence of the methanogenic community structure on the formation of methane from root derived carbon in the rice rhizosphere

The intermediates produced by fermentative bacteria during the degradation of rhizodeposits may be used further by methanogenic archaea for the formation of methane (Dannenberg and Conrad, 1999; Lu et al., 2005). Our results indicate a link between the composition of the methanogenic community of the rhizosphere, the amount of ^{13}C-labeling for porewater acetate, and the methane emission rate originating from recently root derived carbon, respectively **(Figure 3.20)**, rather than the absolute abundance of methanogenic archaea.

The composition of the methanogenic community in the different microcosms was investigated by illumina sequencing analysis. The number of reads was different with respect to the different soil-systems, while the number of observed OTUs, predicted coverage (Good's coverage), species richness (Chao1 estimator), and diversity (Shannon index) were almost the same in all microcosms, as well as in the different tested soil compartments **(Table 3.4)**. All samples revealed at least 99 % of the methanogenic community composition according to the sample coverage. It could furthermore be assumed that a higher number of reads would not result in a further increase of the samples coverage in all of the compartments and microcosms **(Figure 3.17)**. Therefore we suppose to have covered the essential part of the methanogenic community structure in order to assure reliable results.

However, colonization of the rhizosphere by methanogenic archaea differed between the rhizospheric soil and the root surface and differed also with respect to the tested soil-systems **(Figure 3.18 and 3.19)**. The rice root surfaces of all microcosms were preferably colonized (more than 50 %) by *Methanobacteriaceae*. Within this family the genus *Methanobacterium* (OTU_6 and OTU_8), as well as OTU_3 which could not be further taxonomically classified, showed to have the

highest abundance on the root surface. *Methanoregula* showed a high abundance on the root surface but only in microcosms based on the mixed inoculum soil-system (OTU_1), as well as in the control (OTU_33). Considering this, in all microcosms based on the different soil-systems the root surface was mainly colonized by hydrogenotrophic methanogens. This could be confirmed by previous studies, in which methanogens colonizing the rice root supposedly produce CH_4 mainly via hydrogenotrophic methanogenesis (Conrad and Klose, 2000; Chin et al., 2004; Conrad, 2007). This could further indicate that fermentative bacteria colonizing the root surface of plants grown in the different microcosms prefer to degrade rhizodeposits to H_2 and CO_2, rather than to acetate. An important factor for the colonization of roots by hydrogenotrophic methanogens seems to be the availability of H_2, since a generally low H_2 partial pressure in a rice field soil could be a limiting factor for hydrogenotrophic methanogenesis (Conrad, 2007). However, the genus of *Methanocella* is thought to be important for the hydrogenotrophic production of methane on rice roots driven by plant derived carbon (Lu and Conrad, 2005). This was in accordance with our results, but noticeable colonization of the rhizosphere by *Methanocella* only occurred in microcosms based on the rice paddy soil-system and in the control.

Nevertheless, *Methanocella* (OTU_34) was found to colonize the entire rhizosphere of all microcosms except the ones based on digested sludge, from which they were absent in the rhizospheric soil but were present in the soil before planting. Therefore, *Methanocella* may play a role in the hydrogenotrophic methane production in the rice rhizosphere of all microcosms. Nevertheless, methanogenic archaea colonizing the rhizospheric soil mainly belonged to the genera of *Methanosarcina* and *Methanosaeta* in all microcosms except in those based on digested sludge. In the rhizospheric soil of the digested sludge soil-system *Methanobacteriaceae* were found to be the dominant methanogenic archaea. A reason for the low abundance of other groups of methanogenic archaea may be the very high abundance of *Methanobacteriaceae* in the digested sludge soil-system before planting. However, hydrogenotrophic methanogens also seemed to be the key players in the rhizospheric soil of these microcosms. This could further indicate that bacteria colonizing the rhizosphere of the digested sludge soil-system in general had a preference for the degradation of rhizodeposits to H_2 and CO_2 rather than to acetate. Whether hydrogenotrophic or acetoclastic methanogenesis was more important in the rhizospheric soil of the other tested soil-systems is uncertain because of the high abundance of *Methanosarcina*, which are able to perform both pathways for production of methane (Liu and Whitman, 2008). Nevertheless, the colonization by methanogenic archaea that are able to perform acetoclastic methanogenesis was higher in the rhizospheric soil compared to the root surface in all microcosms except those based on digested sludge.

4.4.1 Link between methanogenic groups and the emission of methane originating from root derived carbon

All previous assumptions only apply for the formation of total methane. To go further into detail, we could identify OTUs of methanogenic archaea whose abundance was somehow linked to the emission of methane originating from recently assimilated root derived carbon **(Figure 3.20 and 3.21)**. *Methanosaeta* (OTU_2), *Methanobacteriaceae* (OTU_3 and OTU_8), and *Methanocella* (OTU_34) were found to be associated with the emission of $^{13}CH_4$. Furthermore, the methanogenic community composition of the rhizosphere was also linked to the amount of ^{13}C-labeling of acetate in the porewater. However, considering several biotic and abiotic parameters, it could be shown that neither plant factors like plant height and plant biomass, nor the organic carbon content of the different soil-systems, had an effect on the methanogenic community structure of the rice rhizosphere.

Microcosms based on the rice paddy soil-system and digested sludge soil-system showed the highest emission of methane originating from root derived carbon. Regardless of whether rhizospheric soil or root surface, both the rice paddy soil-system and the digested sludge soil-system possessed the highest abundance of the OTUs linked to $^{13}CH_4$ emission. In microcosms based on the mixed inoculum soil-system on the other hand, $^{13}CH_4$ emission was low and the abundance of almost all of the OTUs linked to $^{13}CH_4$, both in rhizospheric soil and on the root surface, was lower than or within the same range as in the other microcosms.

Methanosaeta are known to use acetoclastic methanogenesis as their obligatory pathway for methane production (Liu and Whitman, 2008) and could furthermore be linked to the emission of $^{13}CH_4$ in this study. Acetoclastic activity in the rhizosphere by *Methanosaeta* is quite plausible, since acetate is a primary fermentation product of the degradation of rhizodeposits (Conrad and Klose, 2000), and can reach millimolar concentrations in the root surrounding area (Conrad, 2007). Species of *Methanosaeta* are able to grow under very low acetate concentrations, whereas species of *Methanosarcina* are more sufficient when the acetate contraction is high (Jetten et al., 1992). In our experiments, acetate in the porewater of the microcosms barely reached concentrations of about 0.3 mM during the reproductive plant stage **(Figure 3.5)**. Therefore, acetate originating from the degradation of rhizodeposits and initial soil organic carbon might be rapidly consumed after formation by various microbial processes. Acetate concentrations were lowest for the digested sludge soil-system and similar between the rice paddy soil- and mixed soil-system. Therefore, different colonization patterns of *Methanosaeta* for the rhizosphere of the

microcosms might not be explained by the acetate threshold concentration but by the formation rate of acetate, if at all.

Furthermore, *Methanosaeta* linked to $^{13}CH_4$ emission showed a higher preference for colonizing the rhizospheric soil compared to the root surface. This applies at least to the rice paddy soil-system and the 100 % rice paddy soil control, while their abundance was almost at the same level in the rhizospheric soil and on root surface in the other microcosms. The contribution of acetoclastic methanogenesis to the total emission of methane was assumed to take a higher standing in the rhizospheric soil compared to the root surface. The higher abundance of *Methanosaeta* in the rhizospheric soil as well as their link to the formation of $^{13}CH_4$ may further indicate the acetoclastic formation of methane from rhizodeposits might be more important in the rhizospheric soil of the rice paddy soil-system compared to the root surface. *Methanosaetaceae* were suggested as the predominant methanogenic archaea in the rice rhizosphere and substantially involved in the conversion of root derived carbon by (Zhu et al., 2014). Furthermore, it was suggested in previous studies with similar microcosms based on rice paddy soil (Pump et al., 2015) that *Methanosaeta* prefer to colonize rhizospheric soil rather than the root surface.

OTU_3 and OTU_8 belonged to the family of *Methanobacteriaceae* and were also related to the $^{13}CH_4$ emission. OTU_8 could be further classified as *Methanobacterium*, while OTU_3 could not be assigned at genus-level. Members of the *Methanobacteriaceae* generally produce methane using H_2 and CO_2 (Liu and Whitman, 2008). Furthermore, *Methanobacteriaceae* linked to the formation of $^{13}CH_4$ showed a preference for colonizing the root surface rather than the rhizospheric soil. This applies at least to the rice paddy soil-system, while their abundance was almost at the same level in the rhizospheric soil and on the root surface in the other microcosms. The contribution of hydrogenotrophic methanogenesis to the total emission of methane was assumed to be of higher importance on the root surface compared to the rhizospheric soil. Also previous studies suggest that methanogens colonizing the rice root are assumed to produce CH_4 mainly via hydrogenotrophic methanogenesis (Conrad and Klose, 2000; Chin et al., 2004; Conrad, 2007). The higher abundance of *Methanobacteriaceae* which could be linked to the formation of $^{13}CH_4$ may furthermore indicate the hydrogenotrophic formation of methane from rhizodeposits might also be more important directly on the roots of rice plants in the rice paddy soil-system compared to the rhizospheric soil.

However, the abundance of *Methanobacteriaceae* in the entire rhizosphere was highest in microcosms based on the digested sludge soil-system. Since members of the *Methanobacteriales*

were found to incorporate root derived carbon preferentially when high concentrations of H_2 were provided by fermentative bacteria feeding on rhizodeposits (Lu and Conrad, 2005), H_2 concentrations in the rhizosphere of microcosms based on digested sludge might have been higher than in the other microcosms.

The genus of *Methanocella* are described as obligatory hydrogenotrophic methanogens (Sakai et al., 2014) and could furthermore be linked to the $^{13}CH_4$ emission in this study. *Methanocella* are known to colonize the rice roots especially (Großkopf et al., 1998b) and also play a key role in the formation of methane from root derived carbon (Lu and Conrad, 2005). In our experiments *Methanocella* linked to the $^{13}CH_4$ emission could not be detected with a noticeable abundance in the rhizosphere of microcosms based on the digested sludge soil-system. Members of *Methanocella* were found to preferentially incorporate plant derived carbon when low concentrations of H_2, which were generated by fermentative bacteria feeding on rhizodeposits, were available (Lu and Conrad, 2005). Therefore, the H_2 concentration could have been a driving factor for the colonization of the rhizosphere by *Methanocella* and *Methanobacteriaceae*, as well as for their contribution to the formation of methane originating from rhizodeposits. Furthermore, the presence of H_2 might have been higher directly on the root surface than in the rhizospheric soil of the rice paddy and mixed inocula soil-systems, while the availability of H_2 was assumed to be equal in the entire rhizosphere of microcosms based on the digested sludge soil-system

In general, *Methanocella* are thought to colonize the rhizosphere and contribute to the $^{13}CH_4$ formation because they seem to have a rather high resistance to aeration (Sakai et al., 2014). This may be a result of the fact that their genome consists of many different genes involved in the detoxification of oxygen species, including catalase and superoxide dismutase (Erkel et al., 2006; Lü and Lu, 2012).

However, previous studies suggested that *Methanocella* and *Methanosarcina* (Lu and Conrad, 2005) or *Methanosaetaceae* (Zhu et al., 2014) are more active on the rice roots than other methanogenic archaea and mainly assimilate photosynthesis-derived carbon. These studies also performed $^{13}CO_2$ pulse-labeling and were based on root incubation or also on planted soil microcosms. Nevertheless, any comparison with our results has to be drawn with caution, because the duration of these experiments was shorter than in our experiments. Direct contribution of photosynthesized CO_2-carbon to the emission of CH_4 is changing with the vegetative stages of rice

growth and furthermore the role of partially decomposed root tissues is thought to be more important at later plant growth stages (Minoda and Kimura, 1994). Therefore, this might also affect the microbial community composition as well as their link to the emission of methane originating from rhizodeposits. However, OTUs according to *Methanocella* and *Methanosaeta* were also identified to be linked with the emission of $^{13}CH_4$ of our results, for all different initial microbial communities available for colonization of the rice rhizosphere. Furthermore, our results also indicated that representatives of the *Methanobacteriaceae* contributed to the formation of methane from root derived carbon in all of the tested microcosms. Nevertheless, we could neither exclude *Methanosarcina* are active on the rice roots nor that they are important for the formation of methane from rhizodeposits. Our results, merely indicate that the abundance of representative OTUs from *Methanosaeta*, *Methanobacteriaceae*, and *Methanocella* could explain the difference in the $^{13}CH_4$ emission rate of the different tested microcosms.

4.4.2 The rhizosphere of rice as a distinct habitat for the colonization by methanogenic archaea

The composition of the methanogenic community in the rice-planted microcosms changed with respect to their abundance in the different compartments: (i) soil before planting, (ii) rhizospheric soil and (iii) root surface **(Figure 3.23)**. This indicates that the overall rhizosphere of rice was a distinct habitat not only for bacterial colonization but also for methanogenic archaea. Furthermore, different methanogenic OTUs were found to prefer the different parts of the rhizosphere for colonization.

Also at OTU-level, the hydrogenotrophic methanogens of *Methanobacteriaceae* (OTU_6, OTU_3, OTU_8, and OTU_24) and *Methanoregula* (OTU_1 and OTU_33) showed a preference for colonizing the root surface across the entire data. In contrast to this, the obligatorily acetoclastic *Methanosaeta* (OTU_21 and OTU_95) and potentially acetoclastic *Methanosarcina* (OTU_11), as well as *Methanomassiliicoccus* (OTU_22) primarily colonized the rhizospheric soil of the microcosms. This also indicates a preference for colonization of the rice root surface by hydrogenotrophic methanogens, as well as for colonization of the rhizospheric soil by obligatorily and potentially acetoclastic methanogens, regardless of the initial microbial community available for colonization in both cases.

Hydrogenotrophic *Methanocella* (OTU_34), *Methanobacteriaceae* (OTU_305), and also acetoclastic *Methanosaeta* (OTU_2) appeared consistently in all soil compartments. Some

representatives of the *Methanobacteriaceae* (OTU_4 and OTU_7) showed a strong association with the soil before planting and gave almost no evidence for colonizing the rhizosphere at all. Possible reasons for this might be: (i) that these groups had no need for spatial proximity to the rhizodeposits; (ii) were outcompeted by other methanogenic archaea which can use root derived carbon more efficiently; (iii) there metabolic activity was suppressed by bacteria using other energetically more favorable electron-acceptors like NO_3^-, Fe(III) or SO_4^{2-}.

OTUs that are ubiquitous in rhizospheric soil and/or on the root surface of all microcosms may be assumed as distinct methanogenic community shaped by a rice plant when present in the initial microbial community available for colonization. *Methanobacteriaceae* (OTU_3, OTU_305, and OTU_8) and *Methanosarcina* (OTU_11) occurred in the entire rhizosphere, as did *Methanosaeta* (OTU_2) but latter only on the root surface. Furthermore, *Methanocella* (OTU_34) were found to be present in the rhizosphere of all microcosms but not on the root surface in microcosms based on the digested sludge soil-system. OTU_3 and OTU_8 (*Methanobacteriaceae*) had a very low abundance in the soil before planting in some of the soil-systems but clearly colonized the rhizosphere.

However, all methanogenic OTUs which could be linked to the emission of $^{13}CH_4$ were found to belong to this distinct rice root associated community of methanogenic archaea. Therefore, the distinct methanogenic community on rice roots shaped by the plant seems to contribute to the formation of methane originating from root derived carbon, as well.

4.5 General discussion and outlook

Rice roots represent the primary site of interaction between the plant and the soil microbial community (Sessitsch et al., 2011). The plants impact extends to the rhizosphere, where microorganism-mediated processes are influenced by the plant root (Hiltner, 1904). Besides many other groups of microorganisms, rice roots are colonized by fermenting bacteria (Ikenaga et al., 2003), as well as by methanogenic archaea (Großkopf et al., 1998b). This implies that the rhizosphere of rice possesses a syntrophic microbial community, which is able to perform degradation of organic matter to methane (Conrad and Klose, 2000).

Methanogenic activity in the rhizosphere comes into focus because rice roots release several carbon compounds, which serve as readily available substrates for the microbial degradation to methane (Dannenberg and Conrad, 1999; Lu et al., 2005; Pump and Conrad, 2014). The microbial

community on the rice roots has been investigated before and likewise the role of certain methanogenic and bacterial groups for the degradation of rhizodeposits to methane. In this study we focused on the influence of the rice plant on different initial soil communities by shaping a distinct microbial community structure in the rhizosphere via the release of root derived carbon. Therefore, we analyzed the patterns of colonization in the different compartments of the rhizosphere by bacteria and methanogenic archaea across different initial microbial soil community structures. Furthermore, we examined the impact of the distinct microbial community of the rice root on the degradation of root derived carbon to methane. In order to get an overview of general patterns of rice root colonization as well as of the structure of a distinct community of the rice rhizosphere, it was necessary to expose the rice roots to different initial microbial communities in defined plant-soil microcosms. Based on (Pump and Conrad, 2014; Pump et al., 2015), we used inoculated soil model-systems as initial seed banks for microbial root colonization, while simultaneously minimizing the different soil properties in combination with a $^{13}CO_2$ pulse-labeling approach, in order to follow the translocation of plant fixed carbon into the rhizosphere.

Nevertheless, it was necessary to extend this established method to use other inocula besides rice-paddy soil. We modified the sand-vermiculite amended microcosms by increasing the amount of inoculum from 10 % to 20 % and using 1.5 kg of soil-system instead of 0.75 kg. Furthermore, we tested different methods of stimulating the methanogenic activity. Our results suggested that stimulation by addition of xylan and cellulose (1:1, 1.0 g kg^{-1}) preferably increased the potential emission of methane and simultaneously maintained the natural composition of the microbial communities of the different inocula better than did an addition of rice straw. The planted microcosms provided the conditions for reliable plant growth and photosynthetic activity, producing $^{13}CH_4$ at the same time as well as ^{13}C-labeled precursors for methane formation, which were exclusively formed from freshly released rhizodeposits. Besides the plant, several other factors were noted, which affect the microbial community in the rice rhizosphere. Edaphic variables, especially pH value (Fierer and Jackson, 2006; Nunan et al., 2005), soil type (Girvan et al., 2003; Fierer and Jackson, 2006), and soil structure (Siciliano et al., 2001; Nunan et al., 2005) have an influence on the structural and functional diversity of the methanogenic community in the rhizosphere (Conrad et al., 2008; Pump et al., 2015). In general, the most important factors influencing the CH_4 production in rice paddy soils include soil type, rice variety, temperature, soil redox potential, and the amount of organic carbon and nitrogen (Conrad, 2002). With our approach we could use different inocula to provide different initial microbial communities available for root colonization, while simultaneously minimizing the influence of the different properties of the inocula. For our microcosms we could show that neither plant factors, like plant height and plant

biomass, nor abiotic factors of the different soil-systems, like initial soil organic carbon- and nitrogen content or pH value had an effect on the microbial colonization of the rhizosphere.

With this modified model-system it was possible to investigate the microbial colonization of the rice roots, as well as their contribution to the formation of methane originating from recently released rhizodeposits in defined plant-soil microcosms.

However, each species is thought to select a specific microbial root community as their root microbiome (Kowalchuk et al., 2002; Bais et al., 2006), and it is noted that the type of the rice cultivar also plays a role for the colonization of the rhizosphere (Conrad et al., 2008). In addition to rice, other wetland plants are thought to be linked to the emission of methane (Joabsson and Christensen, 2001; Ström et al., 2003; Saarnio et al., 2004). Therefore, it may conceivably prove beneficial to investigate other plant species of other submerged ecosystems and rice varieties to get an insight into the general link between root colonization and the contribution of root derived carbon to the emission of methane.

Although the fermentation pattern of root released carbon apparently does not differ much from that of soil organic matter, the microbial community involved seems to be completely different (Conrad and Frenzel, 2002). We could identify different bacterial and methanogenic OTUs which were linked to the formation of methane from rhizodeposits across the different microcosms. Furthermore, the degradation of rhizodeposits to methane was found to depend on the bacterial and methanogenic community composition, but not on their absolute abundance in the rhizosphere. In summary, the contributions of functional bacterial groups in the rice rhizosphere to the formation of methane from root derived carbon are thought to be versatile.

While some genera like *Bradyrhizobium* seem to have a positive effect on the release of degradable carbon compounds by the root, others like *Kineosporiaceae* and *Bacteroidales* seem to be actively involved in the degradation of rhizodeposits to precursors for methane formation. Nevertheless, bacteria which contribute to $^{13}CH_4$ emission and were ubiquitous in the rhizosphere of all different soil-systems could not be identified. Therefore, further investigation with our approach of methanogenic environments which contain *Bradyrhizobium*, *Kineosporiaceae*, and *Bacteroidales* may close this gap. In general, many anaerobic environments might be qualified for this, but since many current studies about such potential environments do not give a detailed

insight into the bacterial community composition up to the necessary taxonomic level, investigations preceding this might be necessary.

However, the microbial community directly colonizing the rice root surface is thought to be more involved in the fermentation of root derived carbon compared to that of the rhizospheric soil (Hernández et al., 2015). We could also detect some tendency as of this effect across all of the microcosms. Nevertheless, this trend was thought to be more important in the microcosms based on rice paddy soil compared with the other soil-systems. An insight into the functional groups involved in the degradation of rhizodeposits might be obtained by a stable isotope probing (SIP) approach for RNA labeling. This method was already used by (Lu and Conrad, 2005; Hernández et al., 2015) and would help to identify microorganisms using the plant root derived carbon. We already designed and started such experiments parallel to the pulse-labeling but skipped this approach. The reason for this was that the total emission of methane was relatively low in the rice paddy soil-system, while the labeling of methane was low in the microcosms based on the mixed soil-system. Hence, a sufficient labeling for the RNA of methanogenic archaea was assumed to be impossible since this group is at the end of the syntrophic degradation of rhizodeposits.

Methanogenic archaea colonizing the rice root are assumed to produce CH_4 mainly via hydrogenotrophic methanogenesis (Conrad and Klose, 2000; Chin et al., 2004; Conrad, 2007). As an extended view our results indicated that the root surface was mainly colonized by hydrogenotrophic methanogens in all of the microcosms, while the abundance of methanogenic archaea which are able to perform acetoclastic methanogenesis was generally higher in the rhizospheric soil of all microcosms except those based on digested sludge. Furthermore, hydrogenotrophic methanogens linked to $^{13}CH_4$ emission also seemed to have a higher abundance on the root surface compared to the rhizospheric soil in most of the soil-systems, but were equally abundant in these two compartments in microcosms based on digested sludge. In general, most of the methanogenic archaea which contribute to the formation of methane from rhizodeposits belong to the hydrogenotrophic ones. Besides *Methanobacteriaceae* and *Methanocella* we were able to link the acetoclastic *Methanosaeta* to $^{13}CH_4$ emission. The contribution of those acetoclastic methanogens to the emission of methane was assumed to be more important in the rhizospheric soil compared to the root surface, at least in the rice paddy soil-system.

Methanocella (Lu and Conrad, 2005) and *Methanosaetaceae* (Zhu et al., 2014) have already been suggested to be more active on rice roots than most other methanogenic archaea and mainly assimilate photosynthesis-derived carbon from plants grown in rice paddy soil. Nevertheless, our

results are not trivial since our conclusion considered the shape of a distinct rhizospheric community linked to the degradation of rhizodeposits to methane across different initial soil communities. In fact, our findings indicate that the overall rhizosphere of rice plants is a distinct habitat for the colonization by bacteria and methanogenic archaea regardless of the initial microbial community structure. Furthermore, all methanogenic OTUs which could be linked to the emission of methane originating from root derived carbon also belonged to this distinct rice root associated community. Therefore, the distinct methanogenic community on rice roots shaped by the plant seems also to contribute to the formation of methane originating from root derived carbon.

Since the composition of root exudates differs from plant species to plant species, each species is thought to select a specific microbial root community as its root microbiome (Kowalchuk et al., 2002; Bais et al., 2006). Additionally, our findings suggest that the specific microbial root community of rice plants may be selected by the preference for root derived carbon for degradation to methane. Nevertheless, the microbial community structure of the rhizosphere was also found to be dependent on the initial pool of microorganisms available for colonization.

This study focused on the colonization of the rhizosphere, rather than on the microbial activity which might provide a link to the conversion of rhizodeposits to methane. Hence, the abundance of specific microbial groups might be similar in the different tested soil-systems, but the activity for the degradation of root derived carbon or general contribution to the emission of methane from rhizodeposits might be different. This is a result of the fact that even if the functional diversity of microorganisms in different soils may be similar, plant factors can affect the activity and proliferation of microorganisms under different conditions (Graystone et al., 1998). Therefore, further analysis based on the RNA of bacteria and methanogenic archaea might be worthwhile to consider.

Methane-oxidizing bacteria are also metabolically active in the rhizosphere (Gilbert and Frenzel, 1998; Frenzel, 2000) and may directly feed on methane originating from rhizodeposits. Therefore, an investigation of the methanotrophic community by an analysis based on the functional *pmoA* marker gene might also provide evidence concerning the link to the oxidation of root derived methane. Since CO_2 originating from the oxidation of methane can serve again as a precursor for hydrogenotrophic methanogenesis (Dannenberg and Conrad, 1999), it is possible that the methanogenic community structure was also affected.

Furthermore, metagenomic approaches have been established in order to to analyze the functional characteristics of microbial communities associated with rice roots (Sessitsch et al., 2011). The advantage of this technique is that it is culture independent and might therefore also target microorganisms which have not been cultured yet (Handelsman, 2004). This might have been of minor importance for methanogenic archaea and methane-oxidizing bacteria since their marker genes already revealed their ecological function, but rather for bacteria in general. Previous approaches focused for example on hydrolytic enzymes, involved in plant-polymer degradation, like cellulases, xylanases, cellobiohydrolases, cellulose-binding proteins, and pectinases (Sessitsch et al., 2011), which might also contribute to the degradation of rhizodeposits to precursors for methanogenesis.

However, the combination of $^{13}CO_2$ pulse-labeling with high throughput illumina sequencing, as well as quantitative PCR, revealed novel and significant insights into the phylogeny and function of microbial key players for the degradation of root derived carbon to methane in the overall rhizosphere of rice plants.

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Supplemental material

Table S2.1 | List of M13-tagged barcodes used with a universal barcode-system. The barcodes were attached to the bacterial 16S rRNA- and *mcrA* gene amplicons for illumina sequencing.

barcode name	barcode sequence (5' to 3')	barcode + M13 sequence (5' to 3')	inoculum	soil compartment
barcode 1-M13	AACGTTCG	AACGTTCGCACGACGTTGTAAAACGAC	rice paddy soil_100 %	rhizospheric soil
barcode 2-M13	ACACACAC	ACACACACCACGACGTTGTAAAACGAC	rice paddy soil_100 %	rhizospheric soil
barcode 3-M13	ACACGTGT	ACACGTGTCACGACGTTGTAAAACGAC	rice paddy soil_100 %	rhizospheric soil
barcode 4-M13	ACCACAAC	ACCACAACCACGACGTTGTAAAACGAC	rice paddy soil_20 %	rhizospheric soil
barcode 5-M13	ACCATGGT	ACCATGGTCACGACGTTGTAAAACGAC	rice paddy soil_20 %	rhizospheric soil
barcode 6-M13	ACGTCATG	ACGTCATGCACGACGTTGTAAAACGAC	rice paddy soil_20 %	rhizospheric soil
barcode 7-M13	ACTGCAGT	ACTGCAGTCACGACGTTGTAAAACGAC	mixed inoculum_20 %	rhizospheric soil
barcode 8-M13	ACTGTGAC	ACTGTGACCACGACGTTGTAAAACGAC	mixed inoculum_20 %	rhizospheric soil
barcode 9-M13	AGAGCTCT	AGAGCTCTCACGACGTTGTAAAACGAC	mixed inoculum_20 %	rhizospheric soil
barcode 10-M13	AGCTAGCT	AGCTAGCTCACGACGTTGTAAAACGAC	rice paddy soil_100 %	roots
barcode 11-M13	AGCTCTAG	AGCTCTAGCACGACGTTGTAAAACGAC	rice paddy soil_100 %	roots
barcode 12-M13	AGCTGATC	AGCTGATCCACGACGTTGTAAAACGAC	rice paddy soil_100 %	roots
barcode 13-M13	AGCTTCGA	AGCTTCGACACGACGTTGTAAAACGAC	rice paddy soil_20 %	roots
barcode 14-M13	AGGATCCT	AGGATCCTCACGACGTTGTAAAACGAC	rice paddy soil_20 %	roots
barcode 15-M13	AGTCCTGA	AGTCCTGACACGACGTTGTAAAACGAC	rice paddy soil_20 %	roots
barcode 16-M13	ATATCGCG	ATATCGCGCACGACGTTGTAAAACGAC	mixed inoculum_20 %	roots
barcode 17-M13	ATCGCGAT	ATCGCGATCACGACGTTGTAAAACGAC	mixed inoculum_20 %	roots
barcode 18-M13	ATGGTAGG	ATGGTAGGCACGACGTTGTAAAACGAC	mixed inoculum_20 %	roots
barcode 19-M13	CACAACAC	CACAACACCACGACGTTGTAAAACGAC	rice paddy soil_100 %	soil before planting
barcode 20-M13	CACATGTG	CACATGTGCACGACGTTGTAAAACGAC	rice paddy soil_100 %	soil before planting
barcode 21-M13	CAGTCAGT	CAGTCAGTCACGACGTTGTAAAACGAC	rice paddy soil_100 %	soil before planting
barcode 22-M13	CATGCATG	CATGCATGCACGACGTTGTAAAACGAC	rice paddy soil_20 %	soil before planting
barcode 23-M13	CATGTGCA	CATGTGCACACGACGTTGTAAAACGAC	rice paddy soil_20 %	soil before planting
barcode 24-M13	CCAATACG	CCAATACGCACGACGTTGTAAAACGAC	rice paddy soil_20 %	soil before planting
barcode 25-M13	CCATGGAT	CCATGGATCACGACGTTGTAAAACGAC	mixed inoculum_20 %	soil before planting
barcode 26-M13	CCTAGGTA	CCTAGGTACACGACGTTGTAAAACGAC	mixed inoculum_20 %	soil before planting
barcode 27-M13	CGATATCG	CGATATCGCACGACGTTGTAAAACGAC	mixed inoculum_20 %	soil before planting
barcode 28-M13	CGATCGTA	CGATCGTACACGACGTTGTAAAACGAC	digested sludge_20 %	rhizospheric soil
barcode 29-M13	CGCGTAAT	CGCGTAATCACGACGTTGTAAAACGAC	digested sludge_20 %	rhizospheric soil
barcode 30-M13	CGTACGAT	CGTACGATCACGACGTTGTAAAACGAC	digested sludge_20 %	rhizospheric soil
barcode 31-M13	CGTTGCAA	CGTTGCAACACGACGTTGTAAAACGAC	digested sludge_20 %	roots
barcode 32-M13	CTAGAGCT	CTAGAGCTCACGACGTTGTAAAACGAC	digested sludge_20 %	roots
barcode 33-M13	CTAGCTAG	CTAGCTAGCACGACGTTGTAAAACGAC	digested sludge_20 %	roots
barcode 34-M13	CTAGTCGA	CTAGTCGACACGACGTTGTAAAACGAC	digested sludge_20 %	soil before planting
barcode 35-M13	CTCTTCTC	CTCTTCTCCACGACGTTGTAAAACGAC	digested sludge_20 %	soil before planting
barcode 36-M13	CTGAAGTC	CTGAAGTCCACGACGTTGTAAAACGAC	digested sludge_20 %	soil before planting

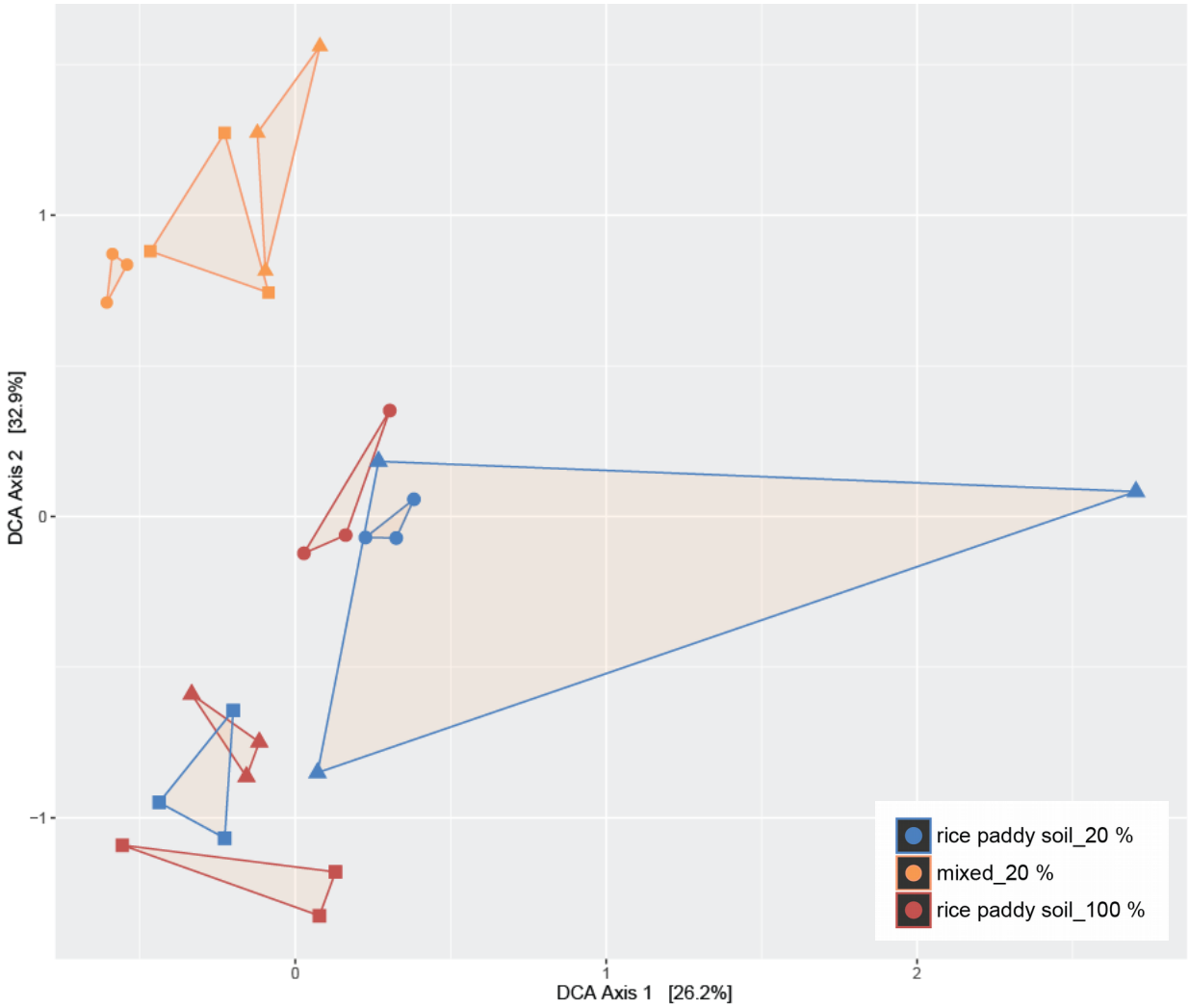

Figure S3.1 | Phylogenetic patterns in the bacterial community structure of rice-planted microcosms with different soil-systems, during the reproductive growth stage. Community similarities were calculated based on OTU-level and plotted by DCA for samples without those from the digested sludge microcosms. Symbols indicate different compartments of the microcosms: soil before planting (■), rhizospheric soil (▲), and root samples (●).

Abgrenzung der Eigenleistung

Das Hauptthema 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 Manuskripts ausgewertet. Das Verfassen des Manuskriptes erfolgte in Zusammenarbeit mit meine Betreuer Prof. Dr. Ralf Conrad.

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Erklärung

Ich versichere, dass ich meine Dissertation

„Colonization of the rice rhizosphere by microbial communities involved in the syntrophic degradation of rhizodeposits to methane”

selbstständig 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 Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg │ Februar 2017