Microbial Communities in Paddy Fields in the Mekong Delta of Vietnam

Functional and Molecular Diversity

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Abstract

Rice paddy fields are considered to be unique ecosystems. Intensive rice cultivation was developed to increase yield and to meet the need of food security. This practice has many negative effects on the soil ecosystem, such as reduction of soil nutrients, soil and water pollution and increase in soil-borne plant pathogens and a possible reduction of soil microorganism diversity. Alternative management strategies are required to counteract these negative effects to maintain soil fertility. The aims of this thesis are to investigate the microbial community in the rice paddy field to assess the influence of microorganisms on the degree of crop residue degradation and in protecting the next crop against soil-borne plant pathogens, including *Rhizoctonia solani*, and to understand the relationship between microbial diversity and functional groups involved in straw degradation and the inhibition of *R. solani* growth. Furthermore, the effect of intensive rice cultivation on the yield, abundance and diversity of the total bacterial community and on the diazotrophic bacterial community compared with the rice crop rotation system is investigated.

Bacteria isolated from rice stubble with both cellulolytic and combined cellulolytic and chitinolytic activity were phylogenetically linked to distinct microbial groups. Selected bacterial isolates with these functions inhibited *R. solani* growth on agar plates; most of these isolates seemed to be neutral with respect to their effect on rice seed germination and radicle length. There was a positive relationship between straw weight loss and the number of isolates and functional groups. Fungal isolates were more important for straw degradation than the bacteria. The growth of *R. solani* was inhibited when it was inoculated on degraded straw. There was a negative relationship between straw weight loss and the growth of *R. solani*. Finally, crop management practices had a significant effect on both rice production and bacterial community structure. Rice yield from all the rice crop rotations that included maize and/or mungbean was significantly higher than that from the rice monoculture. Besides the yield effect, the structure and diversity of the total bacterial community and of the potential nitrogen-fixing bacterial community were significantly influenced by crop rotation when compared with that detected in the rice monoculture soil.

This thesis highlights that crop rotation systems had a positive impact on rice production and on soil microbial diversity in the rice field ecosystem. Results from this study can be applied in the future development of a sustainable rice management.

Keywords: rice, crop rotation, N_2-fixing bacteria, bacterial community, diversity, cellulolytic, chitinolytic, decomposition, *Rhizoctonia solani*, antagonistic effect.

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Dedication

To my grand-mum, you are waiting for me and my family day by day,
To my parents and my parents in-law,
To my husband Le Tan Trien,
To my son Le Do Minh Thien

Contents

List of Publications

This thesis is based on the work reported in the following papers and is referred to in the text by their Roman numerals:

I Do Thi Xuan, Sadhna Alström, Anna Rosling, Nils Högberg. Phylogenetic and functional characterization of cultured and cloned bacteria inhabiting rice stubble and their potential antifungal effect (manuscript).

II Do Thi Xuan, Sadhna Alström, Anna Rosling, Ingvar Nilsson, Nils Högberg. Generalist microorganisms with combined cellulolytic and chitinolytic activities drive decomposition of rice straw and antagonism against *Rhizoctonia solani* (manuscript).

III Do Thi Xuan, Vo Thi Guong, Anna Rosling, Sadhna Alström, Benli Chai, Nils Högberg (2012). Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa. Biol Fertil Soils* 48(2), 217–225

IV Do Thi Xuan, Qiong Wang, Sadhna Alström, Vo Thi Guong, Anna Rosling, Nils Högberg. Diazotrophic and total diversity of soil bacterial communities in a rice field rotated with mungbean and maize (submitted).

The contribution of Do Thi Xuan to the papers included in this thesis was as follows:

I Participated in planning the experiment and conducted all the laboratory work. Data analysed, results summarized and the manuscript written together with the supervisors.

II Participated in planning the experiment and set up the experiment with the supervisors' assistance. Conducted all the laboratory work. Data analysed, results summarized and the manuscript written together with the supervisors.

III Planned the experiment and collected samples. Conducted all the laboratory work. Data analysed, results summarized and the manuscript written together with the supervisors.

IV Planned the experiment and conducted most of the laboratory work. Data analyses, summarizing results and the manuscript written together with the supervisors.

Abbreviations

Cellu	Cellulolytic activity
Chiti	Chitinolytic activity
Cellu+chit	Combined cellulolytic and chitinolytic activity
SWL	Straw weight loss
f	Functional group
iso	Isolates
RRR	Rice–rice–rice
RMR	Rice–maize–rice
RMgR	Rice–mungbean–rice
RMgM	Rice–mungbean–maize
VN	Vinh Nguon
HA	Hoa An
C	Carbon

1 Rice cultivation in Vietnam

Vietnam is located between the latitudes 8° and 24°N and the longitudes 102° and 111°E. It covers a total area of approximately 331,210 km^2. The population in 2012 is estimated to be 91.5 million inhabitants. The northern regions have a humid subtropical climate, with humidity averaging 84% throughout the year, whereas the southern regions have a tropical climate with high humidity and a distinct wet and dry season. Vietnam is the second biggest exporter of rice in the world. Rice is mainly produced in the Mekong delta, southern Vietnam. The Mekong delta has 1.7 million hectares of rice fields, which produce about 20 million tonnes of rice per annum (Vietnam Statistics 2009).

Due to the favourable environmental conditions for rice cropping, people in this region cultivate two or three rice crops a year. Growing the same crop repeatedly causes a reduction in yield and a build-up of soil-borne pathogens. In addition, intensive chemical inputs (fertilizers and pesticides) may influence soil microbial diversity. The negative side effects of intensive rice cultivation may be reduced with the use of alternative agricultural practices. For this strategy to be successful, the role of microorganisms in these processes must be taken into account. Here, soil microbial diversity, including the functional characteristics of the microorganisms was studied in order to understand the role of communities as well as selected microbes in sustainable rice production.

This study has addressed questions relating to the microbial community that inhabits the rice stubble that is left in the field. The effect of crop rotation on the general soil microbial community as well as on the diazotrophic community is another area that was studied within the scope of this thesis. Enhanced management of microbial communities and crop rotation in rice fields have the potential to protect the soil environment from the negative impact of intensive rice cultivation and maintain long-term soil fertility.

2 Ecology of rice cultivation

Rice (*Oryza sativa* L.) is one of the world's oldest and most important crop species, having been domesticated about 8,000–9,000 years ago. Rice is the main staple food for more than a third of the world's population, about 3 billion people and provides 20% of the human calorie intake (Zeigler and Barclay 2008). Different rice cultivars are adapted to a wide range of environments: such as tropical and temperate climates, lowland and highland regions and a

Figure 1. Rice cultivation in the Mekong delta, Vietnam

wide range of soil types. About 50% of rice is grown under intensively irrigated systems, which accounts for 75% of the global rice production

(Zeigler and Barclay 2008). Recently, the continuous use of chemical fertilizers to enhance crop productivity has been recognized in terms of the negative effect on the complex system of biogeochemical cycles.

The paddy field is a unique agro-ecosystem, where the field is flooded for most of the period of rice cultivation and is left under drained conditions during the off-crop season. The paddy field ecosystem, therefore, consists of diverse habitats for microorganisms in time and space, such as aerobic/anaerobic soil conditions, floodwater, rice roots, rice straw stubble and composted materials. In addition, gradients from stagnant to percolating water provide environments with different oxygen levels. These habitats are abiotically different microenvironments that could exhibit biologically distinct properties. Such heterogeneity of the habitats should influence the structure and diversity of microbial communities in the paddy field ecosystem as a whole and may support various microbiological processes occurring in paddy fields, most of which are agronomically and biogeochemically important (Kimura 2000; Kirk 2004).

Rice production in the Mekong delta is divided into two agro-ecosystems: irrigated and rainfed areas. In the irrigated area there are 4 major rice cultivation systems, i.e. 3 rice crops per year, 2 rice crops + 1 upland crop and 2 rice crops, 2 rice crops + fish/shrimp cultivation. In the rainfed areas four different systems of rice production have been practiced: single traditional rice (transplanting rice crop), single medium rice crop of high yield varieties with 110- 140 days of growing crop, 2 rice crops (1 traditional crop + 1 medium rice) and 1 rice + fish/shrimp (Sanh et al. 1998). The introduced, modern, high-yield rice varieties can produce up to 10 tonnes ha^{-1}; however, the amount of fertilizer required for reliable yield is very high. Urea is the most common N source for rice with a recommended application rate of about 100 kg urea-N ha^{-1}. However, farmers normally apply an overdose of fertilizer, using up to 180 kg N ha^{-1} in some regions to maintain yields. However, the efficiency of urea in rice paddy fields is often very low, generally around 30%–40%, and in some cases even lower (Choudhury and Kennedy 2005). The low N-use efficiency partly contributes to the emission of greenhouse gases such as nitrous oxide, nitrite oxide and ammonia (Choudhury and Kennedy 2005).

2.1 Crop management

Intensive cultivation of the same susceptible host plant stimulates specific plant pathogenic organisms (Janvier et al. 2007). To reduce the disadvantages of intensive cultivation, crop rotation and the application of beneficial biological control agents to the field are examples of alternative routes for a sustainable

agriculture. The Romans developed the crop-rotation system over 2000 years ago to maintain and improve soil fertility, with nitrogen-fixing legumes as an integral component (Palacious and Newton 2005). Rotating crops with non-host or less susceptible plants may cause a decline in the specific pathogenic population due to their natural mortality and the antagonistic activities of other organisms (Kurle et al. 2001). Larkin and Honeycutt (2006) studied the effects of crop rotations on Rhizoctonia diseases of potato and found that these were reduced for most rotations compared with that in potato monoculture. Mendes et al. (2011) recently found that Actinobacteria, Alpha- and Beta-Proteobacteria increased in their abundance in soil suppressive to *R. solani*. Dodor and Tabatabai (2003) showed that multicropping systems, including maize–soybean–maize–soybean, maize–maize–oat–meadow, maize–maize–oat–meadow and maize–oat–meadow–meadow rotations, enhanced the activities of the amidohydrolases (amidase, L-asparaginase, L-aspartase and L-glutaminase) in the soil compared with that found in soil undergoing continuous monocropping of maize and soybean. Acosta-Martinez et al. (2004; 2010) found higher levels of soil enzyme activity (arylsulfatase, β-glucosidase and β-glucosaminidase) in soils undergoing crop rotation involving growth of cotton compared with soil under continuous cotton cultivation. In addition, Dung (2011) studied the diversity of the actinomycetous community colonizing rice straw residues in cultured soil undergoing various crop rotation systems in the Mekong delta, Vietnam. He found that crop rotation systems affected the actinomycetes and that a rice monoculture system decreased actinomycetous diversity.

2.2 Soil-borne pathogens in rice and biocontrol

The rice disease profile has changed over the years in response to changing rice cultivation practices, such as the increasing use of direct seedling and the planting of new high-yield cultivars. These changes are due to a reduction in arable land area, rapid population growth and the need for greater efficiency and productivity in agriculture. As yields increase, greater amounts of nitrogen fertilizer are applied in intensive rice production systems. The excess nitrogen leads to a luxuriant vegetative growth and a dense crop canopy that favours disease development (Mew et al. 2004).

Sheath blight, caused by an aerial form of *Rhizoctonia solani*, is one of the newly emerged rice diseases that are threatening the stability of rice production. Sclerotia and mycelium are two forms in which *R. solani* can survive and infect the plant host (Kobayashi et al. 1997). Sclerotia can survive in soil and crop residues for a long period due to the protection derived from

the heavy melanized outer cell layer, whereas the mycelium survives in plant debris. *R. solani* can infect a rice plant at any growth stage, including the seedling (Gangopadhyay and Chakrabarti 1982), panicle and booting or flowering stage (Sharma and Teng 1990; Cu et al. 1996). According to Mendes et al. (2011), sheath blight caused by *R. solani* is an economically important fungal pathogen because of its ability to cause both pre- and post-damping off in many crops, including rice. Groth (2008) studied the effects of cultivar resistance on rice sheath blight, yield and quality and found that rice yield loss ranged from 8% in the moderately resistant cv. Jupiter to 40% in the very susceptible cv. Trenasse.

Mycelia of this fungus contain chitin in the cell wall. The application of antagonistic chitinolytic bacteria and/or fungi offers an alternative strategy to the use of synthetic chemical pesticides. Chitin consists of unbranched chains of beta-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) and is widely distributed in nature (Li 2006). The presence of chitinolytic activity in bacteria and fungi has been shown to be an unexploited potential application in biological control against soil-borne plant pathogens (Nielsen and Sørensen 1999). A wide range of organisms, e.g. virus, bacteria, fungi, insects, plant and animals, produce chitinases (Li 2006). In this study, we focused on bacteria and fungi capable of hydrolysing chitin. In bacteria, this trait is activated to digest chitin for utilization as a carbon and energy source whereas chitinolytic activity in fungi is thought to have autolytic, nutritional and morphogenetic roles, as well as roles in competitive interactions among fungi (Li 2006).

Biological control of soil-borne pathogens is often attributed to improving the nutrition that boosts host defences or directly inhibits pathogen activity and growth. Antagonistic microorganisms, e.g. *Pseudomonas* spp., *Bacillus* spp. (Wiwattanapatapee et al. 2007), *Burkholderia* sp. (Cuong et al. 2011) and Trichoderma spp. (Khan and Sinha 2006) have been used to control disease caused by *R. solani*. The potential of microorganisms for biological control can result from one or more mechanisms. For example: 1) the inhibition of microbial growth by diffusible antibiotics and volatile organic compounds (VOCs) and toxins (Berg 2009), including the production of diacetylphloroglucinol (DAPG) and of hydrogen cyanide, a common antifungal agent produced by *Pseudomonas* (Ahmad et al. 2008); 2) competition for colonization sites, nutrients and minerals with pathogenic agents, for example, bacteria with the ability to solubilize and sequester iron and phosphorus from the soil such as *Pseudomonas* spp., *Enterobacter*, *Erwinia* (Babalola 2010); and 3) parasitism, which may involve the production of extracellular cell-wall-degrading enzymes, such as cellulase, chitinase, β-1,3-glucanase, protease and

lipase, which can lyse cell walls (Muleta et al. 2007) and suppress deleterious rhizobacteria, as reviewed by Babalola (2010).

2.3 Molecular methodology to assess microbial community

The meaningfulness of studies about the diversity and structural composition of microbial communities relies on the methodological tools used. Traditionally, the methods used to analyse soil microorganisms have been based on cultivation and isolation (van Elsas et al. 1998). A wide variety of culture media has therefore been designed to maximize the recovery of diverse microbial groups. Culture-based methods are limited because only a small proportion of the microbes in soil are accessible to study.

Advances in molecular technology have accelerated the development of cutting-edge techniques to study soil microbial communities. These techniques are generally DNA-based methods, which have provided deeper insights into the composition and structure of microbial communities compared with the culture-based methods. For the successful use of these new methods, the development of primer pairs that target the conserved region of the 16S ribosomal RNA (rRNA) or their genes (rDNA) from the environment are considered to represent useful ecological markers for prokaryotes, for cloning and for microbial community fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer 1999) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997; Marsh 1999). Complex molecular fingerprints of microbial communities can be obtained using these methods by direct extraction of the soil DNA and polymerase chain reaction (PCR) amplification of the DNA markers of the community of interest. Not only can these techniques be used to analyse both cultured and uncultured microorganisms but they are also rapid and, therefore, can be used to determine changes in community structures in response to different environmental factors. Besides the total community, the structure of specific subgroups can also be assessed (Garbeva et al. 2006). In recent years, the rapid development of next-generation sequencing technologies such as 454 pyrosequencing has allowed vast numbers of partial 16S rRNA genes from uncultured bacteria to be sequenced. In addition to bypassing previously needed cloning and/or cultivation procedures, with their associated biases, community structures can now be investigated at a much higher resolution by revealing taxa that are much less abundant. The 454 pyrosequencing approach has been used to investigate a wide range of bacterial communities by targeting different variable regions of the 16S rRNA genes. Examples of variable regions are the V4 region, which is used to detect bacterial communities in

rhizosphere soil of biofuel crops, corn, canola, soybean, sunflower and switchgrass (da C. Jesus et al. 2010), the V6 region in deep-marine biospheres (Huber et al. 2007) and the *nifH* region in global marine surface waters (Farnelid et al. 2011). In this study, the total 16S rRNA bacterial communities and the N_2-fixing bacterial communities were explored using 454 pyrosequencing to target the V4 and *nifH* regions, respectively. The 454 pyrosequencing datasets were processed according to the flow chart illustrated in Fig. 2.

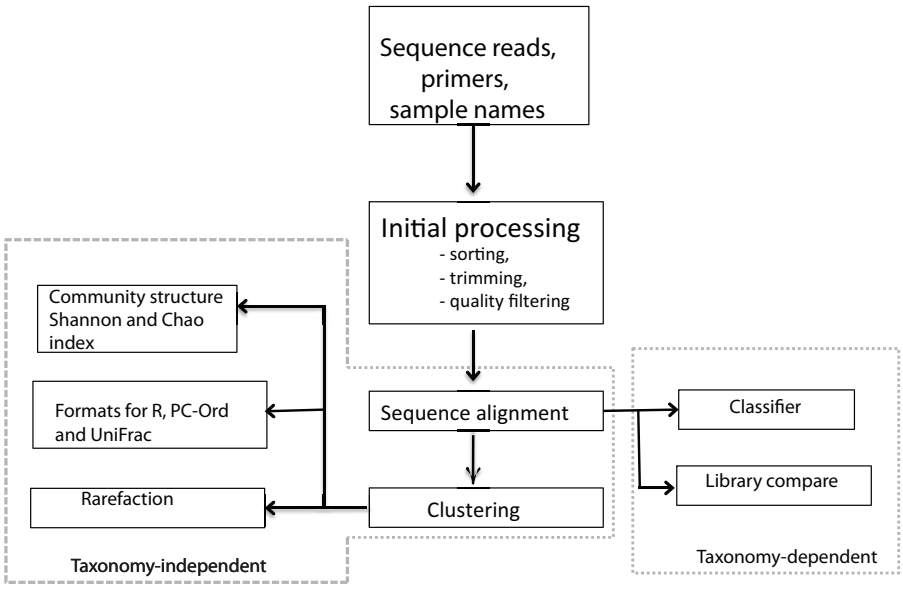

Figure 2. Flow chart showing how the 16S rRNA and *nifH* pyrosequencing data was processed in the RDP pipeline.

2.4 The microbial community in paddy field soils

The bacterial communities in paddy soils have been investigated using both cultivation-independent and cultivation-dependent molecular techniques (Chin et al. 1999; Groβkopf et al. 1998; Henckel et al. 1999; Janssen et al. 1997). Kimura et al. (2001) reported Gram-positive bacteria as major decomposers of rice straw that was incorporated into paddy soil microcosms under submerged conditions. By contrast, both Gram-negative bacteria and fungi were found to be responsible for the decomposition of leaf sheaths and blades under oxic conditions in upland soils (Nakamura et al. 2003). RNA stable isotope probing revealed that the bacteria actively assimilating C from pulse-labelled rice plants were *Azospirillum* spp. (Alphaproteobacteria) and members of the

family Burkholderiaceae (Betaproteobacteria). These organisms were present in high abundance in the rice root environment (Lu et al. 2006). Asakawa and Kimura (2008) compared bacterial community structures in different habitats in a Japanese paddy field ecosystem by comparing the DGGE profiling data and they found that dominant bacterial communities were different in diversity and stability, and phylogenetically distinct from each other in their respective habitats. Matsuyama et al. (2007) and Sungano et al. (2005) studied the bacterial community in plant residues in a Japanese paddy field using DGGE and T-RFLP. They found that members of Firmicutes (clostridia), Alpha-, Gamma-, Delta-proteobacteria, Nitrospira, Acidobacteria, Bacteroidetes, Verrucomicrobia and Spirochaetes were the predominant microorganisms in the rice residues. In addition, Tanahashi et al. (2005) reported the presence of members of these groups during the decomposition of rice straw compost when incorporated into flooded paddy field soil.

Besides degrader communities contributing to the C pool in rice paddy fields, free-living nitrogen-fixing bacteria substantially contribute to the N pool in natural ecosystems. Biological dinitrogen fixation is considered to be the second most important biological process on earth after photosynthesis (Zuberer 2005). Microorganisms that can utilize inert atmospheric N as their own nitrogen source are called diazotrophs (Zuberer 2005). This process provides a suitable alternative for the development of sustainable agriculture, satisfying human needs and conserving natural resources at the same time (Giller and Cadisch 1995; Vance 1997). Previously, very few bacterial species were considered to be nitrogen fixers (Postgate 1982). Young (1992) has documented that nitrogen fixation is a property that can be found in representatives of most of the bacterial phyla and also among methanogenic Archaea. Rodrigues et al. (2004) found a strain of *Verrucomicrobium* isolated from termite guts that revealed nitrogen fixation genes. Two years later, Romero (2006) further documented 6 major lineages or phyla within the domain bacteria having nitrogen-fixing members: Proteobacteria, Cyanobacteria, Chlorobi (green non-sulfur), Spirochetes, Gram-positive bacteria (Firmicutes and Actinobacteria). The application of next-generation sequencing has enabled a greater number of taxa with a potential N_2-fixing gene to be detected. Wartiainen et al. (2008) reported the genetic diversity of free-living N_2-fixing bacteria in paddy soil based on *nifH* gene sequences, and assessed their contribution to the N input in the rice paddy ecosystem.

N_2-fixing microbes can exist in symbiotic association with a host or without hosts, free living in soil (Zuberer 2005). A paddy field is a habitat for numerous groups of diazotrophs (Ariosa et al. 2005; Kennedy et al. 2004; Ladha and Reddy 2003). Some studies have investigated microorganisms from

the rice rhizosphere that can increase rice yield, such as plant growth promoting rhizobacteria, which act as bio-fertilizers (Cong et al. 2009; Mirza et al. 2001). Many of these microorganisms are beneficial not only as biological control agents against rice fungal pathogens but also in terms of improved seed germination and seedling vigour (Mew et al. 2004). Mew and Rosales (1986) performed *in vitro* tests with non-fluorescent and fluorescent Pseudomonas bacteria isolated from rice fields, rhizosphere soils, diseased and healthy plants. They found that 91% of fluorescent Pseudomonas isolates inhibited the mycelial growth of the fungal pathogen. In addition, several N_2-fixing microorganisms have been isolated from rice fields (Elbeltagy et al. 2001; Park et al. 2005; Vaishampayan et al. 2001; Xie et al. 2003). Strains of *Azotobacter, Clostridium, Azospirillum, Herbaspirillum, Burkholderia* and Azoarcus, as well as cyanobacteria, have been shown to fix nitrogen, and are suitable for use as bio-fertilizers (Choudhury and Kennedy 2004). Yasmin et al. (2004) reported that Bacillus sp. Z3-4 and Azospirillum sp. Z3-1 isolated from rice fields in Tanzania could improve rice crop productivity.

2.5 Microbial diversity

The most unique feature about Earth is the existence of life, and the most extraordinary aspect of life is its diversity (Cardinale et al. 2012). Biodiversity is the variety of life, including variation among genes, species and functional traits in an ecosystem, and has an impact on the functioning of that ecosystem and, in turn, on the services that the ecosystem provides humanity. It is often measured as: richness, which is a measure of the number of unique life forms; evenness, which is a measure of the equitability among life forms; and heterogeneity, which is the dissimilarity among life forms. It is well known that the species richness and the abundance of each species can influence ecosystem functioning (Cornwell et al. 2008; Niklaus et al. 2006; Reed et al. 2008). Understanding the former relies on accurate species identification, which increasingly is dependent on molecular approaches, especially for microorganisms. Understanding the latter requires a knowledge of the functional role that each species plays in ecosystem processes (such as nutrient cycling) and a way to measure the abundance of each species (Johnson et al. 2009).

In rice cultivation, less than half of the total rice biomass is edible and the remaining parts consist of straw, stubble and rice root. It has been shown in the laboratory that the decomposition rate of the straw residues above ground is faster than that of the roots below ground (Lu et al. 2003). The different decomposition rates are due to both the chemical composition of the residues

and the microbial community involved in degrading these residues. Focusing on biological processes, changes in different residue sources can alter the decomposition process, indicating that understanding the significance of biodiversity on decomposition is essential to assess the consequences of biodiversity change for carbon and nutrient cycles (Hattenschwiler et al. 2005b). Cellulose degradation is one of the most important biological processes because of the large amount of cellulose in plant dry weight (30–50%). This process can occur under aerobic and anaerobic conditions. Both bacteria and fungi are actively involved in this process (Boer et al. 2005). It may be considered unimportant which group of organisms is responsible for the decomposition of the residues in soil; however, bacterial or fungal decomposition can result in different amounts and composition of decomposed products (Fischer et al. 2006). Aerobic cellulolytic fungi are remarkably effective degraders in cellulolytic systems compared with aerobic bacteria (Boer et al. 2005). These two degrader groups can either facilitate, partition or inhibit interactions depending on the substrates they inhabit. Facilitative interactions result in benefits received by one species in the presence of others and niche complementarity is the differential use of resources by different species (Loreau and Hector 2001; Tiunov and Scheu 2005). The partitioning interaction occurs when one group is benefitted while another group has a neutral effect on the interaction. Finally, the inhibition interaction happens when one group gets a benefit and the other is negatively affected as a result of the interaction.

There is now unequivocal evidence that biodiversity loss reduces the efficiency by which ecological communities capture biologically essential resources, produce biomass, decompose and recycle biologically essential nutrients (Cardinale et al. 2012). Biodiversity is declining worldwide, primarily because of human-induced global changes (Lawton and May 1995), and at least some soil species are known to be vulnerable to these changes (Bardgett et al. 2005; Briones et al. 2007; Eggleton et al. 2002; Parrent et al. 2006; Scheu and Schulz 1996). Several studies in agro-ecosystems have reported reductions in soil faunal biodiversity associated with increased management intensity (Adl et al. 2006; Bloemers et al. 1997; Decaëns and Jiménez 2002; Eggleton et al. 2002).

Diverse communities are more productive because they contain key species that have a large influence on productivity, and differences in functional traits among organisms increase total resource capture. Heemsbergen et al. (2004) showed that eight soil macrofauna species (earthworms, isopods and millipedes) stimulated rates of litter decomposition and litter fragmentation by different amounts. Dang et al. (2005) studied litter decomposition by fungal

communities of one to eight species in microcosms. They found that mean mass loss from litter did not differ across the species richness treatments, but communities with greater richness exhibited less variability in litter decomposition, suggesting that this process had greater stability.

3 Objectives of this study

- To investigate the prevalence of microorganisms exhibiting both cellulolytic and/or chitinolytic activities in rice stubbles with respect to their biodegradation ability. To examine if these functional traits contribute to the antagonism of a soil-borne plant pathogen as well as early rice growth stimulation. In addition, specific functions of microorganisms inhabiting rice stubble were linked to microbial phylogeny. **Paper I**
- To understand the relationship between microbial diversity and the ecosystem function of decomposition and the antagonistic process, as well as the relationship between these two functions. **Paper II**
- To study the effects of different crop rotation treatments on the diversity of the soil bacterial community structure and rice yield. **Paper III**
- To explore the impact of different crop rotations on the diversity of the N_2-fixing bacterial community. To investigate the N_2-fixing bacterial community in relation to the total bacterial community. To compare the diversity of the N_2-fixing bacterial community found in a rice paddy field with that found in marine surface waters. **Paper IV**

4 Introduction to papers

Paper I - In rice cultivation, more than half the biomass is inedible. Straw residues, including root, straw and stubble residues, serve as the major carbon source in paddy fields. Straw residues are also considered to be suitable sources of inoculum for soil-borne plant pathogens such as *Rhizoctonia solani* after rice seed harvest (Kobayashi et al. 1997). Incorporating straw residues into the soil helps to sustain soil organic matter levels, improve physical and

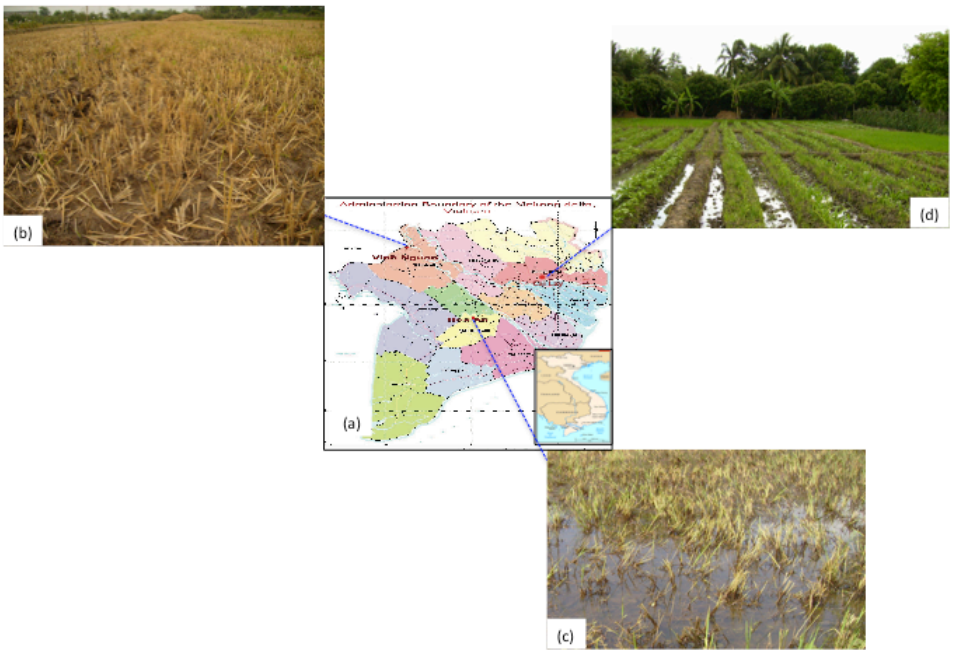

Figure 3. A map of the Mekong delta with locations of three rice fields (a) in the study; Vinh Nguon (b), Hoa An (c) and Cai Lay (d) field (**papers I, III** and **IV**).

chemical properties and increase nutrient availability in paddy field soils (Hadas et al. 2004; Smith et al. 1992). However, the direct incorporation of residues into the paddy field under anaerobic conditions enhances methane production and emission, which contributes to greenhouse gas and global climate change (Denier van der Gon and Neue 1995; Watanabe et al. 1998; Watanabe et al. 1999). Decomposing straw residues before returning them to the soil might be a means of reducing methane emissions and maintaining soil fertility in rice cultivation. The hypothesis in this study is that rice stubble left over in paddy soil may serve as a substrate for beneficial microorganisms. Some microorganisms may enhance the next rice crop by stimulating nutrient cycling through effective decomposition and by suppressing soil-borne pathogens such as *R. solani* that have survived from the previous crop.

Paper II - Diversity is the range of significantly different kinds of organisms and their relative abundance in natural habitats. Changes in microbial diversity can alter the decomposition process (Hattenschwiler et al. 2005a).

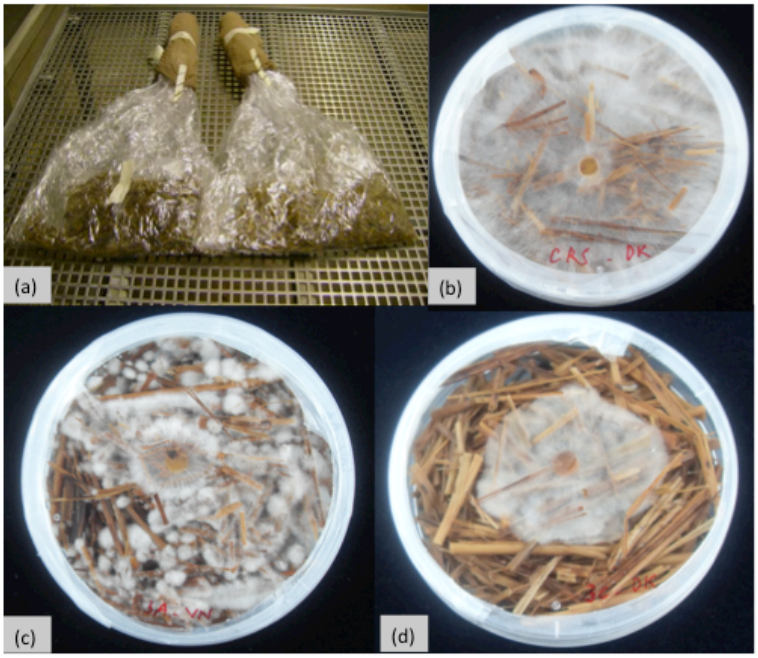

Figure 4. Microbial diversity experiment using 16 microorganism isolates inoculated on rice straw in single or in mixtures of isolates. (a) Microcosm study and degraded straw inoculated with *R. solani;* (b) the control with only *R. solani* growth, (c) *R. solani* growth on the fungus-degraded straw and (d) *R.solani* growth on the bacterium-degraded straw (**paper II**).

Decomposition and the sequestration of organic carbon from straw residues are considered to be important components in ecosystem functioning. Biodiversity

and carbon (C) cycling have been the foci of much research in recent decades, partly because of changes induced by anthropogenic activities, which are likely to continue (Nielsen et al. 2011). However, little is known regarding to what extent the number and function of species play a role in the decomposition process. Therefore, understanding the significance of different basic mechanisms, e.g. the synergistic and antagonistic effects of diversity on decomposition, is essential to assess the consequence of biodiversity change for carbon and nutrient cycling. The hypotheses investigated in this study are that: 1) an increase in the number of isolates would increase the degree of straw weight loss; 2) cellulolytic microorganisms showing more functions, such as chitinolytic or nitrogen-fixing ability, are effective as straw decomposer(s) with a possibility to protect crops from damage caused by the soil-borne plant pathogen *R. solani*; 3) there is a positive relationship between straw weight loss and antagonism.

Paper III - Crop rotation practices have been implemented to increase yield. The positive effect of crop rotation on the abundance of beneficial microorganisms and disease suppression has recently been revealed in several studies (Larkin and Honeycutt 2006; Wardle et al. 2003; Warembourg et al. 2003). Larkin and Honeycutt (2006) found that rotation of the main crop together with other crops increased yield and microbial diversity and also reduced the incidence of *R. solani*. In a sugar beet–*Rhizoctonia solani* pathosystem, disease suppression has been linked to the increased abundance of specific bacterial groups, including Proteobacteria, Firmicutes and Actinobacteria (Mendes et al. 2011). The hypothesis is that different specific rotational crops grown with rice have an effect on bacterial community structure and diversity as well as rice yield in a paddy field.

Paper IV - In paper III, specific rotational cropping with different crops affected changes in the structure and diversity of bacterial community. Dung (2011) found that rotation of a specific crop together with rice effected the composition of Actinomycetes colonizing rice straw left over in the rice field. In addition, Orr et al. (2011) found significant effects of organic and conventional crop rotation on the diversity of total bacterial community and nitrogen-fixing activity. However, to date, the effect of rice crop rotation on the soil N_2-fixing bacterial community in rice fields has not been studied. This study was set up with the aim to better understand the N_2-fixing bacterial community in a rice field rotated with mungbean and maize because this group of crops contribute to the available N source for the coming crop. Our hypotheses are that the abundance of different N_2-fixing phyla changes in different crop rotations in paddy soil, and that a change in the composition of the total bacterial community would reflect the different composition of the N_2-

fixing bacterial community. Furthermore, the N_2-fixing bacterial community in this study is also compared with that in marine environments.

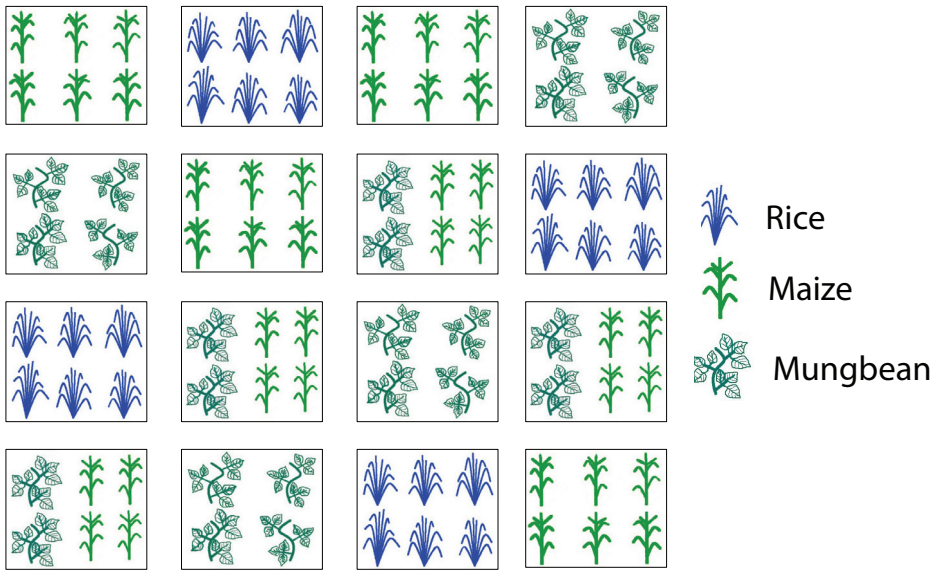

Figure 5. A randomized block design with four treatments and four replicates of each treatment at Cai Lay field, Vietnam (**papers III** and **IV**). Illustration by *Le Tan Trien*

5 Materials and Methods

5.1 Field sites and experimental design

Three paddy fields were selected in the Mekong delta, Vietnam, to study the effects of soil types and crop management systems on both cultured microorganisms and uncultured bacterial diversity. The Hoa An (HA) paddy field (9°45'54" N, 105°35'59" E) had an acid sulfate soil and the Vinh Nguon (VN) paddy field (10°72'54" N, 105°10' E) had an alluvial soil (**paper I**). These paddy fields were cultivated with two rice crops per year. The Cai Lay paddy field (10°34' N, 106°00' E) had an alluvial soil and was the site of a long-term field experiment set up with the aim of studying the impact of specific rotational crops on rice yield and bacterial diversity (**paper III**). In the Cai Lay field, the main crop of rice (*O. sativa*) was rotated with maize (*Zea mays*) and mungbean (*Phaseolus aureus*) crops in different combinations in a cultivation system of three crops per year. This field experiment was set up as a randomized block design with four treatments: (1) Rice–Rice–Rice (RRR), which acted as the control; (2) Rice–Maize–Rice (RMR); (3) Rice–Mungbean–Rice (RMgR); (4) Rice–Mungbean–Maize (RMgM) (**papers III** and **IV**).

Table 1. Summary of soil chemical parameters for the field sites selected in the study

Field	K_{total} (%K)	SOM (%C)	N_{total} (%N)	P_{total} (%P)	$P_{avail.}$ ($mgPkg^{-1}$)	pH (H_2O)
Hoa An (HA)	1.68	8.15	0.45	0.04	5.2	3.64
Vinh Nguon (VN)	1.94	2.99	0.25	0.03	1.3	5.02
Cai Lay	-	4.04	0.24	0.03	4.04	5.5

5.2 Sampling procedures and microorganisms

Stubble and straw residue samples were collected from the first two paddy fields (described above) for **papers I** and **II**. Rice seed was bought from a local agricultural consultant (**paper I**). To study the effect of diversity on straw degradation and antagonism *in vitro* (**paper II**), ten specific bacterial and 6 fungal isolates were selected on the basis of functional characterization of the isolated microorganisms from **paper I**

In the long-term crop rotation experiment (**papers III and IV**), soil samples were taken at two different cropping periods: (1) in May 2007 in the middle of the growing season when rice, mungbean and maize crops were grown, and in February 2008 after the rice seed was harvested in all treatments. These soil samples were analysed in terms of the effects of crop rotation on abundance and diversity in the total bacterial community and with special reference to the N_2-fixing community. In all cases, the soils and rice stubbles collected were stored at 4°C until further processing in the laboratory.

5.3 Investigations on microbial communities

Rice stubble samples collected from the VN and HA rice fields were studied with respect to phenotypic and genotypic characterization. They were isolated and multiplied using standard nutrient media appropriate for bacteria and fungi (**papers I** and **II**). These isolated microorganisms were screened for the functional cellulolytic trait. The cellulolytic isolates were further characterized for their chitinolytic activity but only bacterial isolates were screened for the presence of fluorescence and N_2-fixing genes. **Paper I** reports results from bacterial isolates only. Selected bacteria with either cellulolytic or combined cellulolytic and chitinolytic activity or cellulolytic activity and a potential N_2-fixing gene were selected for further testing to determine their impact on early rice seedling development and on their *in vitro* inhibition of *R. solani* growth (**paper I**).

Genotypic characterization of both cultured and cloned bacteria inhabiting rice stubble was performed by cloning and sequencing of the 16S rRNA region. The primer pairs used to target the 16S rRNA region were 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 907R (5′-CCG TCA ATT CCT TTR AGT TT-3′). Concomitantly, only cellulolytic bacteria carrying a potential nitrogen-fixing gene were identified using nested PCR reactions with primer pairs *nifH3* and *4* in the first round and primer pairs *nifH1* and *2* in the second round. For fungi, the ribosomal RNA gene internal transcribed spacer (ITS) region was targeted by using primer pairs ITS1F (5′CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC-3′)

(**paper II**). All sequences obtained using culture-dependent and culture-independent approaches were arranged into operational taxonomic units (OTUs) based on 98% sequence similarity for further investigation of the phylogeny and taxonomic identity.

Ten bacteria and 6 fungi were selected from **paper I** for further study. They were categorized into 6 different functional groups (Table 1, **paper II)**. Isolates with more than 2 functional traits were defined as generalist and those with only a cellulolytic trait were defined as specialist. A microcosm was designed to study their effect on rice straw decomposition. The microorganisms were inoculated singly or in different mixtures with the purpose of studying the relationships between microbial diversity and the ecosystem function of decomposition. There were 40 different treatment sets and the non-inoculated control treatment with 3 replicates of each. Microcosms were placed in a growth room and spaced well apart from each other and incubated at 30^{o}C for 6 weeks (with a light/dark regime of 12/12 hours). The degree of decomposition was calculated in terms of the dry straw weight loss (SWL) of the decomposed straw compared with the weight of fresh straw before inoculation. Decomposed straw from the microcosms inoculated with all 16 single isolates and with a mixture of all these isolates were inoculated with *R. solani* to explore the antagonistic potential of straw degraders against a soil-borne plant pathogen. Furthermore, the relationship between straw weight loss and antagonism was assessed (**paper II**).

The primer pairs used for 454 pyrosequencing were the V4 FLX forward primer 5′-AYTGGGYDTAAAGNG-3′ (E. coli position 563–577) and the reverse primers were 5′-TACNVGGGTATCTAATCC-3′, 5′-TACCRGGGTHTCTAATCC-3′, 5′-TACCAGAGTATCTAATTC-3′ and 5′-CTACDSRGGTMTCTAATC-3′ (E. coli position 785–802). The forward primer V4 FLX was added with 72 different tags (8 bp each). Rice yield was calculated based on dry weight per hectare when the rice crop was harvested for all four treatments in 2008 (**paper III**).

The 454 pyrosequencing approach was not only used for the total 16S rRNA bacterial community but was also used to study the N_2-fixing bacterial composition by targeting the *nifH* gene using nested PCR of *nifH3* and *nifH4* for the first PCR and PyrB*nifH1* amended with 71 different tags and PyrA*nifH2* for the second PCR (**paper IV**). The N_2-fixing bacterial community was studied in four treatments, RRR, RMR, RMgR and RMgM, and at the two sampling occasions in 2007 and 2008 as described in **paper III**. Due to the failure of short reads during the initial sequencing process in the Fungene pipeline, the *nifH* sequences of the nitrogen-fixing bacterial community in soil samples from the RRR, RMR and RMgR treatments collected in 2007 and

from the RMgM treatment collected in 2008 were excluded from the analyses of both *nifH* and 16S rRNA in this study. The sequences from the remaining replicates for each treatment were pooled together for further analyses.

5.4 Data analyses

In this study, the basic data were analysed by analysis of variance (ANOVA) (**papers I, II** and **III**) to compare soil types and/or treatments affecting bacterial community and diversity. In addition, multiple regression analysis (**paper II**) was also used to establish relationships between decomposition rate and other variables that contribute to the decomposition rate.

With the community structure analyses from the pyrosequencing dataset (**paper III**), multivariate analysis methods were used to fully exploit the data, including non-metric multi-dimensional scaling (NMDS). NMDS is an ordination technique that differs in several ways from nearly all other ordination methods. The advantage of this method is that NMDS does not make any assumptions about sample distribution so it is well suited for a wide variety of data. NMDS also allows the use of any distance measure of the samples, unlike other methods that specify particular measures, such as covariance or correlation in PCA (principal component analysis) or the implied chi-squared measure in detrended correspondence analysis (Holland 2008). However, multivariate analysis alone does not give a statistical measure of the differences between samples. Multi-response permutation procedure (MRPP) and indicator species analysis (ISA) can give a meaningful framework for the findings.

UniFrac is a program suite for computing differences between microbial communities based on phylogenetic information. This method measures the phylogenetic distance between sets of taxa in a phylogenetic tree as a fraction of the branch length of the tree that leads to descendants from either one environment or the other, but not both. The UniFrac analysis can be used to assess overall differences in community structure based on phylogenetic distance and is very sensitive to the changes in richness, evenness and genetic diversity and the composition of communities in the environments (Lozupone and Knight 2005). This is essential to move beyond pairwise significance tests. Although sequences are often used to catalogue the types of microorganisms present in a single environment, comparisons between sequences from multiple environments are increasingly important because they can test whether microbial community composition changes in response to specific environmental variables.

6 Results

6.1 Functional activities of microorganisms inhabiting rice stubble based on culture-dependent analysis (**paper I**)

The cultivation approaches used in this study resulted in the isolation of a total of 259 bacteria from rice stubbles, 110 of which exhibited cellulolytic activity. Among the cellulolytic isolates, 19% showed combined cellulolytic and chitinolytic activity and less than 6% carried *nifH* genes. Fluorescent activity was found to be absent among the tested bacteria. The proportion of bacteria that exhibited combined cellulolytic and chitinolytic activity at the HA site was significantly higher than that at the VN site ($p<0.05$).

Among the isolates with cellulolytic, combined cellulolytic and either chitinolytic or N_2-fixing potential activity, 30 bacteria were selected and used to inoculate rice seed to evaluate their impact on germination and radicle length. Out of these 30 isolates, 13% were deleterious whereas the remaining isolates were apparently neutral in their effect on both seed germination and radicle length (Fig 3, **paper I)**. Parallel to the rice seed germination tests, the results from an *in vitro* antagonism assay against *R. solani* showed that 31 out of 32 tested isolates with cellulolytic or combined cellulolytic and either chitinolytic or N_2-fixing potential activity significantly inhibited the mycelial growth of *R. solani* (Fig 2, **paper I)**. However, we did not find any relationship between the tested functional characteristics and the two processes.

6.2 Diversity effect on rice straw decomposition and antagonistic processes (**paper II**)

In the study to determine the effect of microorganisms on rice straw decomposition in microcosms, the effect of their inoculation on degrees of SWL was evident. SWL was nil or insignificant in non-inoculated microcosms.

Both diversity and isolate-dependent effects were observed. Results from the multiple regression analyses showed that five fungi; *Stachybotrys bisbyi* (Sta), *Fusarium* sp. (Gib), *Sarocladium oryzae* (Sar), *Dendryphiella* sp. BR354 (Den) and *Rhizomucor variabilis* (Rhi), one bacterium *Burkholderia gladioli* (*Bur*) and increasing the number of isolates in mixtures contributed significantly to rates of SWL (Table 3, **Paper II**). The fungi played a dominant role in straw degradation compared with that of bacteria (Fig 2, **paper II**). Among the effective degraders, the four generalists, Sta, Rhi, Den and Bur, exhibited combined cellulolytic and chitinolytic activity and the two specialists exhibited only cellulolytic activity. There was a positive relationship between SWL and the number of isolates.

The degraded straw from all 16 individual treatments and the treatment with the mixture of all 16 isolates showed different levels of inhibition of *R. solani* growth. However, growth was only significantly inhibited by straw degraded by the generalists: Sta, Rhi, Den, *Bur* and *Bacillus pumilus* (*Ba3*); the specialists: Sar, *Pantoea* sp. (*Pan*) and the mixture of 16 isolates (Fig 3b, **paper II**). We also found a negative correlation between SWL and the growth of *R. solani*.

6.3 Bacterial communities in rice stubble and in rice fields with different crops (**papers, I, III, IV**)

The sequences of the 259 bacterial isolates in **paper I** were classified into 17 families. The bacterial community inhabiting stubble in the HA field (16 families, H′= 2.14) were at least twice as high compared with that in the VN field (7 families, H′= 1.68). The sequences from the isolates with no activity, only cellulolytic and combined cellulolytic and chitinolytic traits were found in specific lineages (UniFrac, $p<0.001$; Fig 4, **paper I**). The number of sequences associated with each of these traits present in the specific lineages were significantly different in their abundance between the two fields (UniFrac, $p<0.001$). Furthermore, results from the Jackknife environment cluster analysis supported the functional lineages with 99.9% after 1000 re-samplings (**paper I**).

Among the cultured bacterial communities, Bacillaceae, Burkholderiaceae and Enterobacteriaceae were the most common families present in the two fields, together ranging up to 62% of the total number of sequences. With regard to the cloned bacterial community, bacterial clones were classified into 47 groups at family or higher taxonomic rank. Bacillaceace was the most common family in the two fields. There were 26 families that were only detected by the cloning approach (Table 2, **paper I**).

The presence of specific rotation crops caused changes in bacterial community structures compared with that found in the rice monoculture based on NMDS analysis (Fig 3, **Paper III**) and Jackknife cluster environment analysis (Fig 2, **paper IV**). Although crop rotation was found to be important as a determinant of shifts in soil bacterial communities, the combination and sequence of rotation crops also resulted in significant dissimilarity in the bacterial community structure. Rice yield from the rotation crop treatments was significantly higher than that from the rice monoculture. It increased by 46% depending on the rotation crop (Fig 1, **paper III**).

We detected up to 18 bacterial phyla in the four crop rotation treatments: RRR, RMR, RMgR and RMgM. Among these bacterial phyla, Proteobacteria, Acidobacteria, Chloroflexi and Verrucomicrobia were the most abundant phyla in all four treatments on the two sampling occasions. The relative abundance of different phyla was significantly different among the treatments on each occasion (Table 3, **paper III**).

The number of unique protein sequences of the four treatments is shown in Table 2 (**paper IV**). At the level of 96% protein identity, the N_2-fixing bacterial community in the four treatments was classified into 12 phyla. The relative abundance of these phyla differed significantly among the four treatments. Proteobacteria and Firmicutes were the most abundant phyla carrying the *nifH* gene (Table 3, **paper IV**). When comparing the relative abundance of the *nifH* community with that of the total 16S rRNA community, the Deltaproteobacteria belonging to the Proteobacteria was the most common group present in both. Although Verrucomicrobia, Chloroflexi and Alphaproteobacteria were major groups of the 16S rRNA community, they represented less than 1% of the *nifH* bacterial community. By contrast, the Firmicutes, Betaproteobacteria and Nitrospira were less abundant (<4%) in the 16S rRNA but members of each of these groups represented between 6% and 35% of the N_2-fixing bacterial community. The relative abundance of Spirochaetes and Archaea phyla compared with that of the total bacterial community was very low or undetectable; however, high proportions (2–7%) of these phyla carried the N_2-fixing gene (Table 3, **paper IV**).

7 General discussions

The findings in this study showed that both soil type (**paper I**) and crop management practices (**papers III and IV**) have an impact on the bacterial community structure in paddy soils. According to Veldkamp (1955) and Mitchell and Alexander (1962), actinomycetes appear to be the most important chitin-degraders in relatively dry, neutral and alkaline soils, whereas unicellular bacteria can become important chitin degraders in neutral or alkaline soil at high moisture levels. Our study investigated stubble samples from two fields with different moisture regimes. The results from **paper I** show that the abundance of bacteria exhibiting combined cellulolytic and chitinolytic activity was more dominant in the HA field than in the VN field. This is indicative of moisture being a determining factor for stubble-inhabiting bacteria with combined functional traits to degrade straw as well as inhibit *R. solani*. The importance of actinomycetes in straw degradation and pathogen inhibition in this study has yet to be elucidated.

7.1 Functional activity of microorganisms

Microorganisms exhibiting functional traits relating to straw decomposition and competition were common in the dead stubble material. In this environment, decomposer microorganisms compete with each other for C sources and it may not be a primary environment to find direct plant growth promoting microorganisms. Most of the selected isolates inhibited the growth of *R. solani in vitro* although we did not see a clear correlation with determined functional characteristics. This may be explained by the PDA medium used for the dual assay test: unlike straw the PDA medium may have provided sufficient nutrients for *R. solani* and bacterial growth and, hence, the bacteria may have used other mechanisms to antagonize *R. solani*. Examples of such mechanisms are cyanide production, siderophore production and antimicrobial

metabolite production (Ahmad et al. 2008). In the Mekong delta, especially in regions where rice is cultivated very intensively, farmers often burn crop residues after the rice seed harvest. This is a way to sanitize the fields before preparing for the coming crop. The alternative practice of adding degraded stubble to the field rather than incorporating fresh materials may reduce methane emissions. The results of this study suggest that the return of degraded stubble by controlled composting instead of burning could be a way to maintain fertility. If the stubble left over in the field is utilized in this way, some of the microorganisms inhabiting rice stubble may not only degrade stubble but also potentially stimulate rice seedling growth by means of nutrient release and disease suppression.

7.2 Diversity effect on straw decomposition and antagonism against *R. solani*

Results from this study showed that not only an increasing number of isolates but also specific isolates influence straw weight loss. Previous studies have focused mainly on cellulose decomposition by inoculation of single fungal or bacterial isolates (Bärlocher and Corkum 2003; Pascoal et al. 2010; Wohl and McArthur 2001; Heemsbergen et al. 2004). The number of cultures used in those studies was fewer than 10 species and showed a positive diversity effect on litter decomposition. The level of diversity was also smaller than that normally encountered in the field, and the communities were created by mixing species from a small pool in different combinations. As a result, those studies do not reflect the way natural communities are assembled (Lepš et al. 2001). In the field environment, species richness is high, which is not only important for the C cycling process but also for other processes that take place concomitantly. Our study is the first to combine different functional characteristics relating to degradation, nitrogen fixation and competition with different species in the diversity experiment (**paper II**). There were 16 isolates used in this study. The different species and functional traits created a gradient in diversity with a different combination of functional traits and number of isolates. It has been shown that specific components of the fungal community in soils take part in the decomposition of particular C sources such as glycine, sucrose, lignin and cellulose (Cox et al. 2001; Hanson et al. 2008). It has also been shown in a laboratory study that the addition of different C sources to soil has a strong effect on microbial community composition (Orwin et al. 2006), suggesting that only certain groups of microbes are associated with the breakdown of specific C compounds. Moreover, cellulose decomposition is one of the processes involved in C cycling and, therefore, any functional trait

related to cellulolytic activity probably directly affects this process. Hemsbergen et al. (2004) studied eight soil macro-fauna species (earthworms, isopods and millipedes) and found that functional dissimilarity among these species drove community compositional effects on leaf litter loss.

Generally, the greater the diversity, the more stable the ecosystem is. Within an ecosystem, reduction of some species may have little or no effect on its entire environment. Regarding specific processes in the ecosystem, loss or reduction of one or more functional species directly influences the ecosystem. In a review, Nielsen et al. (2011) concluded that there was little evidence for a predictive relationship between species richness and C cycling in soil, although they pointed out that the presence/abundance of particularly influential species or functional groups can have an impact on C dynamics, often in a substantial way. In our study, it was seen that both specific isolates and a mixture of all isolates increased SWL. The effective isolates belonged to different functional groups, from high cellulolytic to low cellulolytic activity in the case of fungi, and both cellulolytic and chitinolytic activity for bacterial species. Facilitative interactions may occur among the functions rather than among the isolates. In the microcosms, it was shown that the addition of specific isolates resulted in effective degradation. In the *in vitro* study, we did detect specific species involvement in specific processes; however, this was not tested under field conditions. However, the culture-independent approach could help to understand the abundance of taxa present in the field. Therefore it is important to link microcosm studies to field studies to understand how specific functional traits are involved in C cycling and their distribution in the environment. Therefore, the effective degraders should be tested in the rice field to understand more about their role in straw decomposition and the outcome of their interaction with other indigenous microorganisms, especially plant pathogens, in terms of sustainable rice crop production.

With regard to fungal and bacterial isolates involved in cellulose degradation in our study, it was shown that SWL caused by fungal degradation was significantly greater than that caused by bacterial degradation. These results are in agreement with those reported by Boer et al. (2005) who showed that aerobic cellulolytic fungi are remarkably effective degraders in a cellulolytic system compared with aerobic bacteria. Furthermore, Mille-Lindblom et al. (2006) and Romani et al. (2006) have studied the ability of a bacterial community and 6 fungal species to degrade litter in aquatic microcosm systems. They also found that fungi played a more important role in litter degradation than that played by the bacteria. Several studies that have investigated fungal and bacterial activities in terrestrial ecosystems have shown a general picture of a major niche differentiation between fungi and bacteria:

"*Bacteria are mostly involved in the degradation of simple, soluble substrates whereas fungi are the main decomposers of solid, recalcitrant substrates*" (Buée et al. 2009). We inoculated both bacteria and fungi together but we did not analyse the chemical composition or the microbial community of the degraded straw. It is not known whether these isolates helped or inhibited each other. However, based on the results from SWL caused by single inoculations and by the mixture of fungal and bacterial isolates, we observed that SWL in the mixture treatments seemed to be caused by fungal isolates only. Nevertheless, since the paddy field is submerged most of time during cultivation, the role of fungi may be essential for degrading straw left over in the field for about 7–14 days after rice seed harvest when the field is dry. During the next crop cultivation, when the field is submerged again, bacteria may take over the role of straw degradation from fungi. Future studies that investigate the temporal gradients of the micro-flora that inhabit the crop residues after the grain has been harvested until the next rice crop is planted would shed light on the role of bacteria and fungi in straw decomposition in rice fields.

Our study is also the first to report the inhibition of *R. solani* growth by straw decomposed by single isolates and a mixture of all isolates. Interestingly, among the effective degraders, 3 fungi generalists and 1 bacterium generalist were also strong antagonists of *R. solani*. In addition, the inhibition of *R. solani* growth by the treatment involving the mixture of isolates seemed to be mainly due to the effective degraders in the mixture; however, we do not know how many isolates survived to the end of the antagonistic experiment. Another reason for the negative correlation between SWL and *R. solani* growth may be reduced nutrient availability in the most decomposed microcosms. The results from generalists are interesting and further studies in rice fields are needed to explore their potential as biofertilizers and biocontrol agents in terms of stimulating degradation of crop residues and suppressing soil-borne plant pathogens.

7.3 Bacterial communities in rice fields

Soil microorganisms play an important role in maintaining soil fertility through biochemical processes, especially in intensive agricultural systems. Microbial diversity and activity are sensitive indicators that reflect the sustainability and productivity of terrestrial agro-ecosystems (Cardinale et al. 2012). This study showed that specific crop rotations cultivated with rice caused changes in bacterial communities and bacterial diversity. These results agree with other studies that have reported that soil type and crop management practices mainly

determine the structure of bacterial communities (Clegg et al. 2003; Garbeva et al. 2008; Larkin and Honeycutt 2006; Orr et al. 2011; Steenwerth et al. 2002). In the middle crop rotation, we found that the bacterial community structure under the rice monoculture treatment tended to be distinct from that present under the other crops. This may be explained by: 1) different crops release different root exudates that stimulate different bacterial communities (Herschkovitz et al. 2005; Lerner et al. 2006) and 2) mungbean and maize plants in the RMR, RMgR and RMgM treatments were grown in aerobic conditions whereas the rice plants in the RRR treatment were grown in anaerobic conditions. Interestingly, soil samples collected from the RMgR and RMgM treatments from the middle crop rotation of mungbean showed that the RMgR treatment had a significantly distinct bacterial community structure and higher bacterial diversity and richness compared with that in the RMgM treatment even though rice had been grown as a pre-crop in both treatments. Similarly, analysis of the soil samples collected after rice seed harvest from the four treatments revealed that the bacterial communities were also distinctly different from each other. These results indicate that not only specific crops but also the order of the crop in the crop rotation affects the bacterial community in the rice field. In addition, the bacterial community structure in the four treatments correlated positively with crop species and rice yield. However, we did not find any correlation between bacterial diversity or bacterial richness and rice yield. Future studies investigating specific effects of different crops on soil chemical and physical properties in relation to the microbial community structures and rice yields are needed to understand more about yield increases in crop rotation systems.

Besides the effect of crop rotation on the total bacterial community structure in the four treatments, the N_2-fixing bacterial community structure and composition were also found to be influenced. In this study, the N_2-fixing bacterial community structures and composition in the four treatments were distinct from each other even though soil samples were collected after rice seed harvest from the RRR, RMR and RMgR treatments. These results were consistent with those reported by Orr et al. (2011) who also found that different management systems effected the diversity and activity of free-living N_2-fixing bacteria and total bacteria. Low diazotrophic diversity was observed in the soil samples from the middle crop treatment of mungbean in the RMgM crop rotation. This may be because fertilizer had been applied in the middle crop treatment. In general, the application of chemical N-fertilizers may be one explanation of why biological nitrogen-fixation ability has been lost in many bacterial lineages when not needed (Martinez-Romero 2006).

The limitation of available N for primary production is widespread, not only in agricultural soils and in terrestrial ecosystems but also in marine ecosystems (Vitousek and Howarth 1991). Our analyses of unique protein sequences of *nifH* bacterial communities show that the *nifH* community in our paddy soil contained a greater diversity of potential N_2-fixing bacteria compared with that in global marine water surfaces surveyed by Farnelid et al. (2011) who used the same methodology. Our results are consistent with those obtained by Gaby and Buckley (2011) who constructed and analysed an aligned dataset from different environments and found a higher diversity of *nifH* sequences in the soil than in the marine environment. This finding provides the basis for further exploration of specific N_2-fixing groups representing different ecosystems that are poorly understood.

Straw residues left in the paddy fields support living microorganisms. Matsuyama et al. (2007), Tanahashi et al (2005) and Sungano et al. (2005) applied DGGE and T-RFLP to study the bacterial community in rice residues and they found members of Firmicutes, Gamma-, Alpha-, Delta-proteobacteria, Nitrospira, Acidobacteria, Bacterioidetes, Verrucomicrobia and Spirochaetes in rice residues and rice straw compost incorporated into paddy field soil. Their results agree in general with our results that Bacillaceae, Enterobacteriaceae, Burkholderiaceae and Pseudomonadaceae were the most common families inhabiting rice stubbles by using cellulose-amended medium. Some genera from these families and from Flexibacteraceae, Microbacteriaceae, Oxalobacteraceae, Rhizobiaceae, Rhodospirillaceae and Sphingomonadaceae had cellulolytic or combined cellulolytic and chitinolytic activities, suggesting that members of these groups may be important for straw degradation in the natural environment. We also found members that did not show cellulolytic activity. It is plausible to think that for instance: 1) the fast-growing bacteria may accidentally appear on stubbles; 2) some bacteria may rest as spores on the stubble; and 3) they may be important in utilizing glucose/other nutrient sources from cellulose degraders or parasitizing other microorganisms.

Implementation of 454-pyrosequencing to analyse samples from the rice crop rotation field proved to be a promising way of providing a deeper understanding of soil microbial ecology, e.g. which species are present (richness), how many there are (abundance) and what they are doing (function) (Buée et al. 2009). Earlier studies reported that only some groups were present in paddy soils such as Alpha-, Betaproteobacteria (Ludermann et al. (2000), actinomycetes and Gram-negative bacteria (Kimura and Asakawa 2006). Asakawa and Kimua (2008) found up to 9 phyla: Proteobacteria, Chloroflexi, Chlorobi, Verrucomicrobia, Acidobacteria, Nitrospira, OP10, Cyanobacteria and Actinobacteria; however, using 454-pyrosequencing was able to

differentiate more than 18 bacterial phyla. Besides these phyla, more than 30% of the sequencing reads did not match any already identified taxon. Twenty bacterial phyla were found in the rice soil rotated with maize and/or mungbean. Across the four treatments and two sampling occasions, we found that Proteobacteria, Acidobacteria, Chloroflexi, Verrucomicrobia were the most abundant phyla. Members of Proteobacteria, Acidobacteria and Verrucomicrobia have been reported to be dominant in the rhizosphere of a wide range of plant species e.g. Korean rice (Lee et al. 2011) and biofuel crops such as soybean, canola, sunflower, corn and switch grass (da C. Jesus et al. 2010). Our soil samples originate from the bulk soil, which indicates that these groups form highly dominant communities in both the rhizosphere and bulk soil. However, there is much less information about the role of the Chloroflexi in natural environments. Further study is needed to reveal the role of this group in agricultural fields and to understand more about their role in the microbiology.

The relative abundance of both total 16S rRNA and *nifH* bacterial communities were shown to be affected by crop rotation systems. The numbers of bacteria carrying the nitrogen-fixation gene (*nifH*) were also genetically diverse in the four rotation treatments. Previous studies based on the DGGE method (Hsu et al. (2012); Mårtensson et al. (2009) studied N_2-fixing bacteria in bulk paddy soil and found that Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Firmicutes were the most dominant groups carrying the *nifH* gene. Wartiainen et al. (2008) found that the active N_2-fixing bacterial community belonged to the same groups. Ours is the first study to report the occurrence of many more bacterial phyla carrying the N_2-fixing gene compared with those reported previously. Furthermore, Shu et al. (2012) found that the N_2-fixing bacteria belonging to Gammaproteobacteria were the most dominant followed by Alphaproteobacteria and Betaproteobacteria in decreasing order in bulk paddy soil. In terms of dominance in the soil, the order of these groups was different in our study: Deltaproteobacteria > Betaproteobacteria > Gammaproteobacteria > Alphaproteobacteria. By contrast our finding showed that Deltaproteobacteria was the most important group in the four treatments carrying *nifH* genes as well as in the total bacterial community. This indicates that soil conditions and management practices may determine the structure of these communities.

In addition the relative abundance of the Firmicutes, Betaproteobacteria, Nitrospira, Archaea, Spirochaetes and Gammaproteobacteria phyla was low in the four treatments despite the fact that the members of these groups seemed to contribute substantially to the N_2-fixing bacterial community composition. We did not find any bacteria belonging to Actinobacteria carrying the *nifH* gene

although this phylum was detected by 16S rRNA sequence analysis. Archaea have previously been documented as a common group of microorganisms in paddy soil (Conrad et al. 2008; Ramakrishnan et al. 2001). Archaea were not detected by 16S rRNA but the relative abundance of those carrying the *nifH* gene ranged between 3% and 7% in the four treatments. Recently, Archaea have been reported to be involved in different processes in paddy fields: they are major contributors to ammonia oxidation (Chen et al. 2008) and methane emission (Singh et al. 2012). There is very little information regarding their importance in the N cycle and methane emission. N_2-fixing bacterial communities need to be further investigated by screening for the *nifH* gene DNA in combination with their activity by analysing soil RNA to better understand which bacterial groups play an active role in the N cycle in paddy fields.

8 General conclusions

The results from this study further our understanding of bacterial community structures in paddy fields in the Mekong delta, Vietnam, where the major agricultural activity is rice cropping, and the results may be applicable to other geographic regions as well. The incorporation of potential beneficial microorganisms that inhabit the rice field back into the paddy field plays an essential role in protecting the soil environment by reducing the chemical inputs needed to maintain nutrient levels and increasing microbial diversity, which all contribute to increasing the yields and, hence, reducing poverty.

Our results show that rice stubble is a rich source of microorganisms with cellulolytic and chitinolytic activity and that generally these microorganisms were antagonistic to *R. solani*. When combined in different combinations, it was shown that specific fungal isolates to a large extent explained diversity effects on decomposition. The strong antagonistic effects towards *R. solani* were observed both in single isolates and in the most diverse communities. These results have practical implications in intensive rice cultivation because increasing the degree of straw degradation and inhibiting pathogenic growth by cellulolytic or combined cellulolytic and chitinolytic microorganisms may help to reduce the time needed for field preparation and protect the environment because incorporating decomposed residues could help to reduce CH_4 production. Thus there are potential applications for microorganisms with multifunctional activities that are vey effective in biodegradation and beneficial in terms of increased crop health and harvests. However, attention must be given to the potentially deleterious taxa, which were found in this study in order for this strategy to be successful in rice cropping.

Moving forward from *in-vitro* studies to the field studies, it was also found that alternating the rice crop with specific rotational crops of maize and mungbean in different rotational systems not only increased rice yield but also changed the structure of the bacterial communities in the rice field. Changes in

the microbial community were shown to enhance some beneficial bacterial groups in the rotation treatments, which potentially reduced the inoculum level of soil-borne plant pathogens. Moreover, N_2-fixing bacteria which are considered to be an important group of microorganisms contributing to N availability, were also affected by the rotational cropping system and by the total N in the soil. Understanding changes in the diversity and composition of this group may partly help to reduce the N fertilizer input in the field. When these factors are combined, it is clear that crop management plays an important role in maintaining yield, soil nutrients and soil diversity. A change in any of these factors may directly impact rice productivity and the income of farmers.

Finally, along with the crop management strategies, advances in the biotechnology used in agriculture research is enabling scientists to increase their knowledge of the biology of microbial communities to understand the environment where rice is grown in paddy fields.

9 Future perspectives

Rice fields harbour a tremendous diversity of soil microorganisms, which partly determine yield as well as soil health. Globally, sustainable rice cultivation systems are closely connected to soil biotic and abiotic factors. Any changes caused by these will impact rice production. According to the 'Technical Assistance ReportNam: Climate Change Impact and Adaptation Study in the Mekong Delta', ADB (2009) and The greater Mekong and climate change: biodiversity, ecosystem services and development at risk, WWF (2009), Vietnam has been identified as one of the countries that is likely to be most vulnerable to global climate change and the Vietnamese Mekong delta, the rice bowl of Vietnam, has been identified as susceptible to the influence of extreme climate events and climate variability. Possible changes in the distribution of floods, cycles of wet and dry season precipitation and increases in the salinity intrusion pattern (Quinn et al. 2010) may directly influence agriculture, particularly rice production.

My future studies will thus focus on:

- further investigation of straw-degrading microorganisms in different rice cultivation systems and their role in the health of the next rice crop.
- investigation of saline-tolerant microorganisms and drought-tolerant microorganisms, including bacteria, fungi and arbuscular mycorrhiza that can stimulate rice growth under stress conditions.
- understanding the dynamics of microbial community structures in rice fields affected by global climate change, e.g. salinity intrusion, drought and extended precipitation periods.
- studying methanotroph, methanogen, nitrogen fixation and denitrifier communities in order to estimate greenhouse gas emissions from rice cultivation.

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