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Towards a sustainable rice culture: the role of microbiota

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"Everything that can be counted does not necessarily count;
everything that counts cannot necessarily be counted."

Albert Einstein

To my parents and sister for all their support....

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ABSTRACT

Rice is cropped worldwide, mainly under wetland conditions and conventional management. The use of synthetic chemical compounds (e.g., fertilizers, pesticides), which are known to have negative impacts on the environment, is regarded as a major problem associated with conventional agriculture. To avoid environmental contamination, organic farming, relying on the use of natural compounds (e.g., minerals, compost) and ancient agricultural practices (e.g., crop rotation) to fertilize the soil and avoid plagues, must be encouraged. Organic farming, considered a more sustainable agricultural practice, should progressively replace conventional agricultural management systems.

Bacteria are common soil inhabitants that play important roles not only on the degradation of xenobiotic compounds (e.g., pesticides) but also on the biochemical cycles, crucial in the maintenance of soil fertility, and thus, crop productivity. Soil bacterial communities are known to be shaped by different factors, justifying the importance of studying the influence of the farming type. Studies assessing the influence of different management practices on soil bacterial communities of paddy soils were scarce and motivated the present work. Considering that conventional farming is still the most widely used, it was also important to assess the feasibility of using bioremediation methods in paddy soils to remove molinate, a herbicide used in rice culture protection. Taking into account these problems, two major specific objectives were defined as i) the assessment of the influence of the type and characteristics of the agricultural management on the paddy soil bacterial community composition and functional activity, and ii) the feasibility of molinate bioremediation in paddy soils.

To assess the influence of the type and characteristics of the agriculture management, two independent studies were carried out. In the first, the paddy soil bacterial community composition and functional activity of a conventional system was compared with that of an organic farming field. In the second study, organic paddies, where an alfalfa-rice crop rotation system (two years alfalfa followed by

two years of rice cropping) was implemented, were compared with an uncropped soil. Variations were monitored between two different stages of crop-rotation system (first and second year of rice cultivation) and over the annual rice cycle, and correlated with different abiotic and biotic parameters presumably capable of shaping the bacterial communities. To assess the feasibility of bioremediation, the efficiency of degradation and natural microbiota stability were studied using the intrinsic potential (natural attenuation) or bioaugmentation.

The study of the influence of the type and characteristics of the agriculture management was carried on with samples collected in the Portuguese experimental farm "Bico da Barca", located in the valley of river Mondego. Composite paddy soil samples were collected at different periods of the annual rice cycle (before seeding, during rice growth and after harvesting) and the bacterial communities were characterized using culture dependent and culture independent methods (16S rRNA based Denaturing Gradient Gel Electrophoresis and 454-pyrosequencing). Other physical, chemical and biochemical parameters were determined in parallel. Comparative analyses of the data were supported by multivariate methods.

The factor most affecting the bacterial community diversity, structure and composition was the presence/growth of rice plants. Uncropped soil showed higher bacterial diversity than cropped soils. Some lineages, such as *Chloroflexi* ("Ellin 6529"), *Acidobacteria*-2, AD3, and *Nostocaceae* were apparently outcompeted in cropped soils.

Over the annual rice cycle, functional changes in conventional and organic farming regimens were observed. Variations on the bacterial community structure and composition were observed in the organically farmed paddy soil samples, but not in those under conventional management.

Important microbial functions occurring in paddy soil over the rice cycle were the organic matter degradation, expressed by intense microbial coefficient (qCO_2) and proteolytic activity, before rice seeding, and the N_2 fixing activity, expressed by a high abundance of cultivable diazothophic population, during rice plants growth. The first function was associated with high abundance of cultivable aerobic

heterotrophic populations. However, the multivariate analysis suggested that both aerobic and anaerobic heterotrophs were involved in the proteolytic activity, through bacteria belonging to lineages such as *Sphingobacteriales*, *Rhizobiales*, and *Actinomycetales* and *Bacteroidales* and *Anaerolineae*, respectively. In turn, the increase of the abundance of cultivable diazothrophs over the rice cycle could have been due to bacteria affiliated to *Chloroflexi*-Ellin6529, *Acidomicrobiales*, *Actinomycetales*, *Rhizobiales*, *Rhodospirilales* and *Betaproteobacteria*. Both the proteolytic activity and the increase in the abundance of the diazothrophs may have contributed to the observed increase in the NH_4^+-N soil content over the rice cycle. Another important variation over the rice cycle was the increase of members affiliated to *Nitrospira* after rice harvesting.

The crop rotation stage was observed to be associated with changes in the abundance of bacterial lineages affiliated to potential aerobic and anaerobic heterotrophs. The presence of alfalfa root debris and aeration conditions, in the first year of rice cultivation, favoured the growth of cultivable aerobic heterotrophic populations associated with a high abundance of bacteria affiliated to *Caulobacterales*, *Sphingobacteriales*, *Flavobacteriales* and *Actinomycetales*. On the other hand, in the subsequent year of the crop rotation, the high soil water, total C and available inorganic-P contents, presumably due to soil organic and inorganic amendments and the eventual presence of rice debris, favoured lineages such as *Bacteroidales*, *Chlorobi* and *Anaerolinea*. Such differences may have been responsible for the different aerobic catabolic activity occurring at the 1st and the 2nd year of rice cultivation. The high abundance of aerobic heterotrophs was correlated with the degradation of complex nutrients, while low abundance of these organisms was correlated with the degradation of amino acids, amines and sugars.

Soil microcosm assays were used to assess the feasibility of using natural attenuation and bioaugmentation methods to clean up molinate contaminated paddy soils. Both bioremediation approaches reduced the soil molinate content, although bioaugmentation allowed a higher extent/faster removal than under natural attenuation. In addition, the exogenous degrading culture used in bioaugmentation

assays did not disturb the autochthonous bacterial community. The occurrence of natural attenuation suggests the existence of indigenous microorganisms able to degrade molinate, reflecting the metabolic diversity of the soil and its ability to respond to environmental stimuli.

RESUMO

O arroz é um cereal cultivado mundialmente. Na sua maioria, o arroz é cultivado em campos alagados e recorrendo a métodos de agricultura convencional. O uso de compostos químicos sintéticos (ex. fertilizantes, pesticidas), devido aos negativos impactos que estes têm no ambiente, é visto como o maior problema associado a este tipo de prática agrícola. Para evitar a contaminação ambiental, a agricultura biológica, que se baseia no uso de materiais naturais (ex. adubos de base mineral e composto), e de práticas agrícolas ancestrais (ex. rotação de culturas) para fertilizar o solo e evitar as pragas, deverá ser estimulada. A agricultura biológica, considerada uma prática agrícola mais sustentável, deveria progressivamente substituir as práticas agrícolas convencionais.

As bactérias são importantes habitantes do solo, e têm um papel essencial não só na degradação de xenobióticos (ex. pesticidas), mas também nos ciclos biogeoquímicos, cruciais para a manutenção da fertilidade dos solos, e consequentemente, para uma boa produtividade das culturas. Tem sido demonstrado que as comunidades bacterianas do solo podem ser moldadas por diversos fatores, entre eles o tipo de prática agrícola. Assim, torna-se importante conhecer as variações das comunidades bacterianas que possam estar associadas a determinadas práticas agrícolas. No caso de solos de arrozais, este tipo de conhecimento é escasso, facto que motivou o presente estudo. Considerando que a agricultura convencional é, ainda, a mais usada, é também importante avaliar a viabilidade da utilização de métodos de biorremediação em solos de arrozais para eliminar o molinato, um dos herbicidas usados na proteção da cultura de arroz. Tendo em consideração estes problemas, foram definidos dois grandes objetivos específicos como i) a avaliação da influência do tipo e características da prática agrícola na composição e atividade funcional da comunidade bacteriana de solos de arrozais, e ii) a viabilidade da utilização de métodos de biorremediação para tratar solos de arrozais contaminados com molinato.

Para avaliar a influência do tipo e características da prática agrícola foram realizados dois estudos independentes. No primeiro, comparou-se a composição e atividade funcional das comunidades bacterianas de solos de arrozais sob agricultura convencional e biológica. No segundo estudo, compararam-se solos de arrozais em modo biológico, onde se implementou um sistema de rotação de culturas luzerna-arroz (dois anos de cultivo de luzerna, seguidos de dois anos de cultivo de arroz) com solo não cultivado. As variações entre as diferentes fases do sistema de rotação de culturas (primeiro e segundo ano de cultivo de arroz) e ao longo do ciclo anual do arroz foram monitorizadas e correlacionadas com diferentes parâmetros abióticos e bióticos, presumivelmente capazes de influenciar as comunidades bacterianas. Para avaliar a viabilidade da biorremediação, estudou-se a eficiência de degradação e a estabilidade da microbiota autóctone usando o potencial intrínseco (remediação natural) ou a bio-inoculação.

O estudo da influência do tipo e características da prática agrícola foi realizado em amostras de solo colhidas no campo experimental "Bico da Barca", localizado no vale do rio Mondego. Amostras compostas de solos de arrozais foram colhidas em diferentes períodos do ciclo anual do arroz (antes da sementeira, durante o período de crescimento do arroz e após a colheita) e as comunidades bacterianas foram caracterizadas usando métodos dependentes e independentes de cultivo (eletroforese em gel com gradiente desnaturante e pirosequenciação-454, baseadas no gene ribossomal 16S). Em paralelo, foram determinados outros parâmetros físicos, químicos e bioquímicos. A comparação e interpretação dos dados foram suportadas por métodos de análise multivariada.

A presença da planta de arroz, isto é, o seu cultivo, foi o fator que mais afetou a diversidade, estrutura e composição da comunidade bacteriana dos solos. No solo não cultivado a diversidade bacteriana foi maior do que em solos cultivados. Tal diferença foi devida ao facto de algumas linhagens bacterianas parecerem ter sido menos competitivas em solos cultivados, tais como *Chloroflexi* ("Ellin 6529"), *Acidobacteria*-2, AD3 e *Nostocaceae*.

Ao longo do ciclo anual do arroz foram observadas variações na funcionalidade das comunidades microbianas, tanto em solos sob regime agrícola convencional como regime biológico. Contudo, em solos sob agricultura convencional não se observaram as variações na estrutura e composição da comunidade bacteriana que caracterizaram os solos sob agricultura biológica.

A degradação de matéria orgânica, expressa pelo intenso coeficiente microbiano (qCO_2) e atividade proteolítica, observada antes da sementeira do arroz, e a atividade diazotrófica, expressa pelo aumento da população diazotrófica cultivável, intensificada durante o crescimento da planta, foram as principais funções microbianas que ocorreram nos solos ao longo do ciclo do arroz. A elevada abundância da população cultivável de heterotróficos aeróbios esteve relacionada com a primeira função. Contudo, a análise multivariada sugeriu que quer heterotróficos aeróbios quer anaeróbios podem ter estado envolvidos na atividade proteolítica, através de bactérias pertencentes às linhagens *Sphingobacteriales*, *Rhizobiales*, *Actinomycetales*, *Bacteroidales* e *Anaerolineae*, respectivamente. Por outro lado, o aumento da abundância da população cultivável diazotrófica ao longo do ciclo do arroz pode ter sido devida a bactérias de linhagens como *Chloroflexi*-Ellin6529, *Acidomicrobiales*, *Actinomycetales*, *Rhizobiales*, *Rhodospirilales* e *Betaproteobacteria*. Quer a atividade proteolítica quer o aumento da abundância de diazotróficos podem ter contribuído para o aumento do conteúdo em NH_4^+-N do solo, observado ao longo do ciclo do arroz. O aumento de organismos afiliados a *Nitrospira* observado após a colheita do arroz foi outra importante variação ao longo deste ciclo.

Observou-se que a fase da rotação de culturas se relacionou com variações na abundância de grupos bacterianos de linhagens que incluem organismos heterotróficos aeróbios e anaeróbios. A presença de detritos de raízes de luzerna e as favoráveis condições de arejamento, no primeiro ano de cultivo do arroz, estiveram associados à proliferação da população heterotrófica aeróbia, expressando-se na abundância de bactérias afiliadas a *Caulobacterales*, *Sphingobacteriales*, *Flavobacteriales* e *Actinomycetales*. Por outro lado, no ano

subsequente da rotação de culturas, o elevado conteúdo em água, carbono total e fósforo inorgânico disponível, presumivelmente devido à adição de suplementos orgânicos e inorgânicos ao solo e à eventual presença de detritos de raízes de arroz, favoreceram linhagens como *Bacteroidales*, *Chlorobi* e *Anaerolinea*. Estas alterações podem ter sido responsáveis pelos diferentes perfis de atividade catabólica aeróbia observados no primeiro e no segundo ano do cultivo do arroz. A elevada abundância de heterotróficos aeróbios correlacionou-se com a degradação de nutrientes complexos, enquanto a baixa abundância destes organismos se correlacionou com a degradação de aminoácidos, aminas e açúcares.

Para avaliar a viabilidade de processos de remediação intrínseca ou de bio-inoculação para regenerar solos de arrozais contaminados com molinato, foram realizados ensaios em microcosmos. Ambos os métodos de biorremediação reduziram o conteúdo de molinato no solo, embora a bio-inoculação tenha permitido uma remoção mais extensa/rápida do que a obtida usando a remediação intrínseca. Além disso, a cultura exógena usada no ensaio de bio-inoculação não perturbou a comunidade bacteriana autóctone. A ocorrência de remediação intrínseca sugere a existência de microrganismos indígenas capazes de degradar o molinato, refletindo a diversidade metabólica do solo e a sua capacidade para responder a estímulos ambientais.

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16S rRNA	16S Ribossomal ribonucleic acid
A	Paddy A – organically farmed paddy – under alfafa-rice rotation system (two years alfalfa followed by two years of rice cropping), in particular, is in the third year of crop rotation
ACE	Abundance based Coverage Estimate
aer Amo	Aerobic ammonifiers (log MPN g^{-1}dry soil)
aer Het	Aerobic heterotrophs (log MPN g^{-1}dry soil)
aer N_2	Aerobic diazotrophs (log MPN g^{-1}dry soil)
anae Amo	Anaerobic ammonifiers (log MPN g^{-1}dry soil)
anae Het	Anaerobic heterotrophs (log MPN g^{-1}dry soil)
anae N_2	Anaerobic diazotrophs (log MPN g^{-1}dry soil)
Anammox	ANaerobic AMMonium OXidation
ANOSIM	Analyis of similarity
ANOVA	Two way analysis of variance
ANS	Uncropped soil from paddy A
Arg	$_L$-arginine
Asn	$_L$-asparagine
B	Paddy B - organically farmed paddy - under alfafa-rice rotation system (two years alfalfa followed by two years of rice cropping), in particular, is in the fourth year of crop rotation
BAA	benzoylargininamide protease activity (μmol NH_3 g^{-1} h^{-1})
C	Biotic control - Non-sterile and non-inoculated soil
CAS	Casein-protease activity (μmol tyrosine $g^{-1}h^{-1}$)
CAT	Catalase activity (mmol H_2O_2 g^{-1} h^{-1})
CCA	Canonical Correspondence Analysis
Celob	$_D$-(+) cellobiose
CFU	Colony Forming Units
Ci	Biotic control - inoculated non-sterile soil
CLPP	Community Level Physiological Profiles
CONV	Conventional farming
CsM	Abiotic control - non-inoculated sterile soil spiked with molinate

CsMi	Biotic control - sterile soil spiked with molinate
Cyclod	α-cyclodextrin
DAPI	4,6-diamidino-2-phenylindole
DCA	Detrended Correspondence Analysis
Denit	Denitrifiers (log MPN g^{-1}dry soil)
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRAPC	Direção Regional de Agricultura e Pescas do Centro
E	Evenness[$E = H/\log S$]
Eryth	i-erythritol
FUN	Fungi counts (log CFU g^{-1}dry soil)
Galactur	Galacturonic acid
Galactur	Galacturonic acid
GalLact	$_D$-galactonic acid γ-lactone
Glu	$_L$-glutamic acid
GluAm	*N*-acetyl-$_D$-glucosamine
Gluc	α-D-glucose-1-phosphate
Gly	Glyceraldehyde
Glycog	Glycogen
H	Shannon index [$\Sigma(ni/N)\log(ni/N)$]
HET	Heterotrophs counts (log CFU g^{-1}dry soil)
INT	Iodonitrotetrazolium violet
INTF	Iodonitrotetrazolium formazan
INV	Invertase activity (μmol glucose $g^{-1}h^{-1}$)
Ketobut	α-keto butyric acid
Lac	α-lactose
M	Natural attenuation assay
Mal	Malic acid
Malon	Malonic acid
Malt	Maltose

MEGA	Molecular Evolutionary Genetics Analysis
MetGlu	β-methyl $_D$-glucoside
Mi	Bioaugmentation assay
MOL	Molinate concentration (mg kg^{-1}soil)
MolA	Molinate hydrolase
molA	Gene encoding molinate hydrolase
MPN	Most Probable Number
N.D.	not determined
N_2O	Nitrous oxide
NH_3	Ammonia
NH_4^+-N	Ammonium in soil (mg NH_4^+-N kg^{-1})
NH_4-Min	NH_4^+-N mineralized (mg NH_4^+ $kg^{-1}10d^{-1}$)
Nit	Total inorganic N in soil (mg N kg^{-1})
N-Min	Total inorganic-N mineralized (mg inorganic-N $kg^{-1}10d^{-1}$)
NO	Nitric oxide
NO_3^-	Nitrate
ON4^T	*Gulosibacter molinativorax*
o-OHBenz	o-hydroxybenzoic acid
ORG	Organic farming
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PD	Phylogenetic Diversity
pH	pH in water
Phe	$_L$-phenylalanine
PheEthAm	β-phenyl ethylamine
Pi	Available inorganic-P (mg P kg^{-1})
PLFA	PhosphLlipid Fatty Acid
p-OHBenz	p-hydroxybenzoic acid
Pt	Total available-P (mg P kg^{-1})

Put	Putrescine
qCO_2	Microbial coeficient (µg C-CO_2 mg biomass carbon h^{-1})
QIIME	Quantitative Insights Into Microbial Ecology
RDA	Redundancy Analysis
RDP	Ribosomal Database Project
Ser	$_L$-serine
SOC	Soil Organic Carbon
TAE	Tris(hydroxymethyl)aminomethane –Acetate -Ethylenediaminetetraacetic acid
Thr	$_L$-threonine
TRIS-HCl	Tris(hydroxymethyl)aminomethane-Hydrochloric acid
t-test	Two-sample statistical test
TV	2,5-diphenyl-3-(α-naphthyl)tetrazolium chloride
Tw40	Tween 40
Tw60	Tween 60
Tw80	Tween 80
UPGMA	Unweighted Pair-Group Method with Arithmetic averages
URE	Urease activity (µmol NH_3 g^{-1} h^{-1})
UV	Ultra-violet radiation
Wt	Water content (%)
Xyl	$_D$-(+) xylose

Chapter 1

Introduction

1.1 Soil

Soil is the portion of the Earth surface with a thickness inferior to one meter. This layer is composed by the products of transformation of soil parent material produced over time due to geological, atmospheric and biological transformations (Buscot 2005; Maier and Pepper 2009). Due to its origin and composition, soil is considered the most complex biomaterial on the planet (Young and Crawford 2004). Two major components drive this complexity, the abiotic soil architecture and the biotic diversity. The remarkable physical, chemical and biological heterogeneity among soils globally results from the integration of these components. In spite of such heterogeneity, three phase systems characterize all soils: i) the solid or mineral inorganic phase, ii) the liquid or solution phase and iii) the gas phase or atmosphere. The composition of each of these phases, will define the properties of a given type of soil (Maier and Pepper 2009).

1.1.1 The solid phase

In general, the solid phase represents about 45 to 50 % of the soil volume, and is constituted mostly by minerals. In this phase, the organic fraction represents only 0.1 to 5 % (Maier and Pepper 2009). The major constituents of the mineral fraction are silicon (47 %) and oxygen (27 %), which combined with soil components with low abundance forms a wide variety of minerals with different sizes (sand, silt and clay), depending on the weathering of the parent rock (Buscot 2005; Maier and Pepper 2009). The percentage of sand, silt and clay within a porous medium defines its texture.

1.1.2 The liquid phase

The liquid phase represents an exchange medium and is extremely important for biological activity. Water represents the major component of this phase and due to water capillarity properties and soil porosity, water movement is generally the most

important process involved in the transport of chemicals (e.g., nutrients) and microorganisms. All microorganisms, even those attached to the solid phase, obtain nutrients from and excrete their wastes into the liquid phase. Thus, the soil solution is an ever-changing environment, due not only to its chemical properties, but also to the dynamic influx and efflux of solutes in response to water movement. Hence, the composition of liquid phase is determinant for all biological activities associated with the soil, i.e., both of microorganisms and plants (Lombard et al. 2011; Maier and Pepper 2009).

1.1.3 Soil atmosphere

In well aerated soils, the soil atmosphere has basic composition similar to the air - nitrogen, oxygen and carbon dioxide. Soils with poor aeration, for instance, flooded soils (due to irrigation or heavy rain) present comparatively lower relative proportions of oxygen and altered ratios of oxygen and carbon dioxide (Maier and Pepper 2009). In addition, under both saturated and unsaturated conditions the soil structure has a critical role on the water flow rate, which affects the diffusion of gases into and out of the soil matrix (Young and Crawford 2004).

1.1.4 Role of soil biota in soil: architecture and biogeochemical balance

Soil is also a complex ecosystem, where bacteria, archaea, fungi, algae, protozoa, invertebrates and plants co-habit and interact (Maier and Pepper 2009; Young and Crawford 2004). Plant roots, which, in general, are the plant components belowground, represent an important part of that ecosystem providing nutrients and a beneficial adsorption surface, favouring plant-microbe and microbe-microbe interactions. Soil biota, *i.e.*, all the aforementioned organisms, play an important role in maintaining soil physic- and biochemical properties. Microbial activity has a critical role in the soil architecture, in particular, algae and prokaryotes, which are

responsible for particles aggregation (e.g., gum production), whereas fungal hyphae and plant roots are responsible to held together physically these aggregates. Both, texture and structure affect the movement of water, exogenous components and microorganisms. Therefore, microbial diversity and activity results from the soil characteristics, contributing also to its heterogeneity (Maier and Pepper 2009; Young and Crawford 2004).

Among the diverse microbial populations inhabiting the soil, prokaryotes are the most abundant numerically (Killham 1994). The immense metabolic diversity of these organisms (autotrophs, heterotrophs, phototrophs, chemo- organotrophs and litotrophs and mixotrophs) relies on the use of both organic and inorganic molecules as sources of carbon, nitrogen, energy and electrons (reducing power). Thus, prokaryotes have a pivotal role in the cycling of chemical elements, in particular, of C and N, and also of S and Fe (Emerson et al. 2010; Falkowski et al. 2008; Gaby and Buckley 2011; Ghosh and Dam 2009; Hanson and Hanson 1996; Hohmann-Marriott and Blankenship 2011; Zumft 1997). Other organisms, such as fungi, algae and plants, are also involved in the C cycling. Fungi, which are in general chemoorganotrophic aerobes, play an important role in the decomposition of organic matter (both simple and complex). Algae and plants, which perform oxygenic photosynthesis, are important for the fixation of carbon dioxide, and thus for the organic fraction of carbon in soils.

1.1.5 Interactions between organisms

In soil, microorganisms interact within each other and with macroorganisms. Different interactions are established and classical classifications define these interactions as symbiotic when only one or both of the elements takes benefit of the interaction (commensalism and mutualism, respectively) or parasitic when one organism lives on or in another organism and obtains its nutrients at the expense of its host (parasitism) (Little et al. 2008). Among microorganisms (in particular, prokaryotes) the interaction is mainly cooperative. Different prokaryotes are

involved on the degradation of both organic and chemical recalcitrant products (e.g., lignin, pesticides among others), as well as in the different stages of the biogeochemical cycles. Because microorganisms establish complex metabolic networks, it is difficult to get a straightforward definition of commensalism or mutualism interactions among prokaryotes. Parasitic interactions among prokaryotes are rare, although may occur. An example is given by *Nanoarcheum equitans*, which genome lacks many key metabolic functions and was recently described as the only potential prokaryote parasite, being *Ignicoccus hospitalis* its host (Paper et al. 2007). In addition to these interactions, microorganisms can interact antagonistically with other microorganisms, in general competing for a common resource (e.g., through the production of antimicrobial substances) (Little et al. 2008). These categories exist in a continuum. Thus, a clear distinction or classification of the established relationships among organisms is not always possible.

Microorganisms and plants have also important interactions. Among the most studied are the symbiotic interactions rhizobia-legume, actinorhizal and the mycorrhiza, which are highly specific (mutualism) (Chow et al. 2002; Jones et al. 2007; Pawlowski and Bisseling 1996; Wall 2000; Zahran 1999). A good example of parasitism is the interaction between phytopathogenic bacteria and fungi with plants (Raaijmakers et al. 2009), causing plant disease. Plant protection is another example of interaction. By antagonism bacteria or fungi may hamper the activity of plant pathogens (Azcón-Aguilar and Barea 1997; Kobayashi and Crouch 2009; Little et al. 2008). In summary, these interactions are important for plant nutrition and protection.

1.2 Agriculture

The conversion of native ecosystems into farming areas represents the starting point of agricultural management. This was an important achievement of humankind, but is also among the oldest anthropogenic activities affecting ecosystems.

Before the industrial revolution, traditional agriculture was, in general, enclosed in small farms, which depended exclusively on human manual labour and had low productivity. Thus, the impact of agriculture on the surrounding ecosystems was lower than when fertilizers and pesticides were introduced in the conventional management systems.

1.2.1 Conventional management

Tremendous changes in agriculture practices were brought by industrial revolution. The increase of the human population, related itself with the mechanization of the processes (Matson et al. 1997), imposed changes in the agriculture management. The low productivity of traditional agriculture management was no longer compatible with the world food demand. The factors contributing most to the low productivity of traditional farming are the depletion of soil plant nutrients (mainly nitrogen) and favourable conditions for the overgrowth of weeds and dissemination of pathogens. Consequently, traditional agriculture gave space to the so called conventional agriculture.

In conventional agriculture, primarily, the mechanization of the processes and the improvement of irrigation systems allowed the expansion of the area devoted to agriculture practices. Moreover, the introduction of chemical compounds, such as pesticides and fertilizers, boosted productivity yields by overcoming the nutritional deficiencies of plants as well as protecting plants from plagues, phytopathogens and weeds (Matson et al. 1997). Such procedures permitted also the long-term cultivation in the same field.

1.2.1.1 Major negative impacts of pesticides and chemical fertilizers on ecosystems (macro-scale overview)

Conventional agriculture has negative impacts on agriculture and surrounding ecosystems. Native lands were ploughed to expand the agriculture area and long-term cultivation with intensive tillage and monoculture systems were implemented, contributing to reduce the soil organic carbon (SOC) storage, mainly under dry climate conditions (Al-Kaisi et al. 2005; Ogle et al. 2005; Parton et al. 2005). Furthermore, the addition of synthetic fertilizers (in particular, enriched in N and P) had negative environmental impacts mainly by leaching water soluble molecules (NO_3^-, NH_4^+ and PO_4^{3-}), causing a generalized contamination and eutrophication of aquatic systems. For instance, NO_3^- may have toxic effects on human beings, in particular on infants (Knobeloch et al. 2000) and NH_4^+ and P may induce eutrofication of downward aquatic environments (Wu 1999). By loss of gaseous forms, NH_3 will contribute to enrich the natural ecosystems (e.g., forests, grasslands) in N, due to downwind deposition. In addition, NO and N_2O will contribute to increase the concentration of greenhouse gases in the atmosphere (Galloway et al. 2008; Schlesinger 2009).

Part of the pesticides and chemical fertilizers applied in agriculture are dispersed in the environment mainly by volatilization and/or leaching. Additionally, some molecules may be trapped within very small soil particles, acting as a long-term "sink" of contaminants with a slow but continuous diffusion to the environment (Park et al. 2005; Reid et al. 2000; Young and Crawford 2004). Pesticides are not organism specific, *i.e.*, their toxic effects span different types of organisms and trophic levels (Cochran et al. 1997; Julli and Krassoi 1995; Khan and Thomas 1996; Moraes et al. 2009; Ondarza et al. 2010). Moreover, the majority of the pesticides are not readily biodegradable and have long half-life periods. Many pesticides are recalcitrant (Bromilow et al. 1999; Ghosh and Singh 2009; Nagy et al. 1995) or are degraded into recalcitrant and/or toxic transformation products (Ellis et al. 1998; Pothuluri et al. 1991). The reported contamination of soil and surrounding environments (e.g., surface and groundwater) by pesticides results

from the above mentioned properties (Albanis et al. 1998; Castro et al. 2005; Durand et al. 1992; Gaus 2000; Mabury et al. 1996). The diffusivity of pesticides is a relevant issue for public health authorities because it has been observed that the contamination may be detected at long distances from the source (Albanis and Hela 1998). Given their harmful effects, the application of several pesticides has been intensively evaluated either by the European and American Environmental Protection Agencies. Some pesticides have been forbidden, and others are used under restricted conditions (European Environment Agency, http://www.eea.europa.eu/ and United States Environmental Protection Agency, http://www.epa.gov/pesticides/).

1.2.1.2 Major negative impacts of pesticides and chemical fertilizers on ecosystems (micro-scale overview)

Pesticides may have negative effects on different soil functions and on microbial community diversity and activity. Several reports demonstrated that pesticides may decrease the soil mineralization activity (El-Ghamry et al. 2001), microbial biomass (El-Ghamry et al. 2001; Sheng et al. 2005), the activity of proteolytic bacteria (Sheng et al. 2005) and substrate induced respiration and nitrification (Saison et al. 2009). On the other hand, pesticides may also increase the activity and abundance of N_2 fixing members (Chen et al. 2009; Das and Mukherjee 2000) and of phosphate-solubilizing microorganisms (Das and Mukherjee 2000). Nevertheless, some of the effects on different soil microbial activities (e.g., mineralization, nitrogen fixation, nitrification) are usually short-termed (Das and Mukherjee 2000; El-Ghamry et al. 2001; Saison et al. 2009). However, long-term exposure to some pesticides may lead to the loss of autochthonous members of the soil microbial community. Laboratory-scale studies with soil isolates revealed that pesticide exposure could induce the loss of specific organisms (Adeleye et al. 2004). Despite the potential soil resilience, the elimination of some populations may restrain its functional activity.

The above mentioned alterations combined with the variation on parameters such as SOC and soil organic N may contribute to decrease the soil quality (Gregorich et al. 1994). After more than a century of conventional agriculture, soils exhaustion and the contamination of the environment and of the human food chain claim for alternatives. The scientific community, the authorities and the general public realise that to avoid loss of soil quality and further contamination of native ecosystems it is crucial to establish and improve already existing sustainable agriculture practices (Mäder et al. 2002; Matson et al. 1997; Pimentel et al. 2005; Rosen and Allan 2007). Moreover, soils already contaminated should be treated (Rölling and van Versevel 2002; Vidali 2001). These objectives demand for a thorough understanding of the response of ecosystem to the use of synthetic chemical compounds and intensive agriculture practices is important.

1.2.1.3 Bioremediation processes to treat contaminated soils

Different technologies are available to treat contaminated soils. The simplest approach consists on digging up the contaminated soil and remove it to a landfill, or capping the contaminated areas of a site. None of these methods remediate the soil, simply contributing to move the contamination elsewhere or restrain the contamination in the original site (Vidali 2001). Advanced techniques such as high-temperature incineration or chemical decomposition (e.g., base-catalyzed dechlorination, UV oxidation) reduce efficiently the levels of contaminants. However, these methods are technologically complex, expensive and, in particular incineration, are not well accepted by public (Vidali 2001). Bioremediation is regarded as a good alternative. In these biological processes, microorganisms individually or, more frequently, cooperatively associated, metabolize and degrade the pollutants (Wackett and Hershberger 2001). These processes can often be carried out on site, offer the possibility to eliminate or transform various environmental contaminants into harmless compounds, have relatively low-costs and have generally a good public acceptance.

Soils bioremediation can be achieved by natural attenuation or bioaugmentation. Natural attenuation depends on the ability of the autochthonous community to biodegrade a contaminant, whereas bioaugmentation depends on the introduction of an exogenous microorganism or mixture of microorganisms (consortium) to carry out that task (Vidali 2001; Vogel 1996). The efficiency of both types of process is determined by abiotic and biotic factors (e.g. water content, bioavailability of the contaminant, electron acceptors, nutrients) (Reid et al. 2000; Röling and van Verseveld 2002; Vogel 1996). The capacity to optimize the bioremediation transformations relies on a thorough characterization of the contaminated site before process implementation. When bioaugmentation is the selected method, a possible limitation is that the survival of the exogenous degrading microorganism(s) may be compromised under natural conditions. The competition with the indigenous community, the existence of predators among others may interfere with the exogenous organisms (Thompson et al. 2005; Vogel 1996; Wackett and Hershberger 2001). Despite the low costs associated with these non-destructive processes, when compared to the other remediation methods, bioremediation usually needs long periods of time to be effective. Among the major reasons for such a delay are the large extension of soils contaminated with low concentrations of contaminants (or low bioavailability) and the heterogeneous spatial distribution of contaminants and degraders (Wackett and Hershberger 2001). Although the treatment of contaminated soils is a good solution to avoid the dissemination of the pollutants, prevention should always be preferred to avoid the continuous contamination of agricultural environments and to preserve the ecosystems. Thus, the implementation of environmental friendly farming practices (e.g., organic farming) should be encouraged worldwide.

1.2.2 Sustainable agriculture

In addition to the above mentioned facts, an increasing awareness of the citizens on the benefits of organic farming (Agriculture and Rural Development, European Commission, http://ec.europa.eu/agriculture/organic/download-information_en and Pesticide Action Network, North America, http://www.panna.org/issues/publication/agroecology-and-sustainable-development) has been contributing to its implementation. Different strategies have been used to implement sustainable agricultural practices. Examples are the utilization of transgenic plants, in particular cereals, able to fix nitrogen (Charpentier and Oldroyd 2010) or plague resistant (Romeis et al. 2006). However, the utilization of ancient agricultural practices has better public acceptance. Ancient agricultural practices include the use of green- or animal manure (Ohno et al. 2005), composts or liquid fertilizers (Bernal et al. 2009; Silva et al. 2013), and the use of controlled release nitrogen fertilizers which reduces the N leaching from soils (Peng et al. 2011). All of these have been considered, so far, good alternatives to conventional practices.

Sustainable agriculture also includes practices aiming to increase SOC storage, such as setting aside land from agriculture production, reducing tillage intensities, and through cropping rotation practices, which also restores the soil N and improves water infiltration when legumes are used (Meek et al. 1990; Ogle et al. 2005). In this type of farming, where herbicides are completely eliminated and intensive tillage is diminished, weed suppression becomes an important issue (Liebman and Davis 2000). The crop rotation system, which involves the sequential cropping of different species (Kelner et al. 1997; Ladha and Reddy 2003; Larkin and Honeycutt 2006; Sun et al. 2009; Yin et al. 2010; Zhao et al. 2009) may be a good method to suppress pests and weeds. Because pests are usually plant species specific and different soil managements are used for each crop, crop rotation is an efficient method to avoid or minimize weeds and pests (Liebman and Davis 2000).

1.3 Agriculture soil microbial ecology: major goals and importance

Rich and diverse microbial communities as those of soils are supposed to be able to respond to different types of perturbation (Ekschmitt and Griffiths 1998). As such, the analysis of the prokaryotic communities may give valuable indications about the stress responses of an ecosystem and mirror the history of the microhabitat (Johnsen et al. 2001). The soil prokaryotic communities are known to be influenced by a wide range of biotic and abiotic factors (Table 1.1). The factors contributing most to community alterations may vary among different ecosystems, and there is always a degree of uncertainty regarding the preferential target populations/ functional activities and the interplay among the different variables. It is recognized that the general soil properties may be influenced by long-term vegetation effects (e.g., deposition of plant debris, plant nutrient uptake) (Kowalchuk et al. 2002). Hence, although bulk soil bacterial populations seem to be more influenced by general soil properties than by plant species composition (Kuramae et al. 2012; Ulrich and Becker 2006), it is possible to conclude that bulk soil bacterial communities are slowly modelled by vegetation.

The complexity of the interactions referred to above require a thorough characterization of bulk soils in terms of biotic and abiotic parameters. Only based on such studies it will be possible to get additional insights into the interwoven relationships among organisms of different trophic levels and the environmental factors.

Table 1.1 Examples of abiotic and biotic factors shaping prokaryotic communities in soil.

Factors	Effects on soil microbial communities	References
	Bulk soil	
Soil characteristics	Differences on the bacterial communities were related to soil factors and not to land-use type or plant species diversity	(Kuramae et al. 2011; Kuramae et al. 2012)
	Soil type was the most important determinant of microbial communities assessed by phospholipid fatty acid (PLFA) profiles	(Bossio et al. 1998)
	Soil type effect exceeded that of plant species in bulk soil	(Kowalchuk et al. 2002; Wieland et al. 2001)
	Induced different bacterial response to pesticide application (expression of catabolic genes)	(Ding et al. 2010)
Parent material	Community structure was clearly related to parent soil material	(Ulrich and Becker 2006)
Soil texture	Soil texture induced changes in bacterial communities	(Ulrich and Becker 2006; van Diepeningen et al. 2006)
Carbon content and pH	Major determinants of bacterial community composition	(Ausec et al. 2009)
pH	Low pH induced low bacterial diversity	(Fierer and Jackson 2006; Lauber et al. 2009)
	Low pH induced decreases in the catabolic evenness	(Degens et al. 2001)
	Low pH did not influence bacterial community diversity	(Ausec et al. 2009)
	Affects microbial biomass, community structure and response to substrate addition	(Aciego Pietri and Brookes 2009)
Available P	In buffered soils, available P was the major driver of changes in composition of prokaryotic soil communities	(Kuramae et al. 2011)

Table 1.1 Continued

Factors	Effects on soil microbial communities	References
	Bulk soil	
Water content	Induced bacterial community structure and composition changes	(Asari et al. 2007; Kikuchi et al. 2007; Noll et al. 2005)
Land use	In successional studies, history of land-use was more critical in shaping the composition of microbial communities than vegetation and soil properties	(Jangid et al. 2011)
Organic vs conventional management	The farm management history influenced the structure of microbial community, nevertheless both communities were positively stimulated by organic amendment (increase in microbial biomass)	(Stark et al. 2008)
	Bacterial diversity was not affected by management, but a higher diversity of bacterial functional communities was found in soils from organic than from conventional farms	(Liu et al. 2007)
	Significant differences were determined in the microbial PLFA profiles	(Bossio et al. 1998)
	Organic management induced higher number of bacteria of different trophic groups, species richness in bacteria communities and more resilience to a drying-rewetting disturbance in soil than conventional management	(van Diepeningen et al. 2006)
Arable soil *vs* forest *vs* native grassland	Catabolic diversity was highest under native grassland and lowest under exotic forest	(Nsabimana et al. 2004)
Crop vs pasture	Crop induced lower catabolic diversity, and increased susceptibility to decrease the catabolic evenness under stress conditions (pH, salinity, metals)	(Degens et al. 2001)
	Decrease in soil microbial biomass	(Haynes and Tregurtha 1999)
Crop vs. forest	Communities from cropped soils were less diverse at the phylum level, but in contrast a higher diversity of members within certain phyla was found in cropped than in forest soil.	(Roesch et al. 2008)

Table 1.1 Continued

Factors	Effects on soil microbial communities	References
	Bulk soil	
Planted *vs*. unplanted	Bulk soil bacterial community composition was different (independent of plant species)	(Zul et al. 2007)
	Cropped soils with *Phaseolus vulgaris* did not change the abundance of *Nitrosospira* (cluster 3) members, but affected those affiliated to *Nitrosomonas* (cluster 8), when compared to uncropped	(Junier et al. 2009)
Rhizosphere	Bulk soil bacterial community were different from those of rhizosphere.	(Uroz et al. 2010; Wieland et al. 2001)
	Rhizosphere	
Plant species	Plant species influenced the microbial communities in plant-associated habitats (rhizosphere and rhizoplane)	(Berg and Smalla 2009; Kowalchuk et al. 2002; Wieland et al. 2001)
Management	Bacterial community and mycorrhizae were not affected by soil management or geographic source	(Chow et al. 2002)
Soil parent material	Soil parent material partly induced changes in the bacterial communities from rhizosphere	(Ulrich and Becker 2006) (Gomes et al. 2010)

Soil is known for its functional redundancy (Stres and Tiedje 2006). Indeed, phylogenetically divergent lineages of microorganisms may share the capacity to undertake some metabolic processes, and thus, display identical activities (Table 1.2). In addition, mixotrophy, frequent among microorganisms, allows the use of different sources of energy and carbon under different conditions (Table 1.2). Temporal successions in bacterial communities have been reported in specific soil microhabitats (e.g., in plant debris, rhizosphere, bulk soil), showing the interdependence of different community members and suggesting that different bacterial members may yield distinct roles in the ecosystem (Bastian et al. 2009; DeAngelis et al. 2011; Rui et al. 2009). Not surprisingly, the patterns of succession may vary according to the environmental conditions (e.g., temperature, soil water content, plant growth) (Asari et al. 2007; Junier et al. 2009; Rui et al. 2009; Watanabe et al. 2009). Indeed, those environmental conditions may influence the development of different phylogenetic lineages, although not necessarily of different functional groups. Thus, the phylogenetic lineages responsible for a given activity may vary, depending on the (micro-) environmental conditions. As a conclusion, it is possible to say that soil functional redundancy is, above all, a result of the richness and diversity of the community.

Table 1.2 Examples of bacteria involved in particular metabolic activities contributing to soil functional redundancy.

Functional activity	Phylum (examples of genera)	References
Phototrophic	*Chlorobi* (e.g., *Chlorobium*, *Chloroherpeton*)	(Bryant et al. 2012; Frigaard and Dahl 2008; Hohmann-Marriott and Blankenship 2011; Imhoff 2003; Madigan 2003)
	Chloroflexi (e.g., *Chloroflexus*, *Roseiflexus*)	(Blankenship 1992; Bryant et al. 2012; Hanada et al. 2002; Hohmann-Marriott and Blankenship 2011; Madigan 2003; Pierson and Castenholz 1974)
	Cyanobacteria (e.g., *Nostoc*, *Oscillatoria*, *Synechococcus*)	(Gallon et al. 1991; Galloway et al. 2008; Hohmann-Marriott and Blankenship 2011; Mager and Thomas 2011)
	Firmicutes (*Heliobacterium*)	(Blankenship 1992; Gest and Favinger 1983; Hohmann-Marriott and Blankenship 2011; Madigan 2003)
	Proteobacteria (e.g., *Ectothiorhodospira*, *Rhodobacter*, *Rhodospirillum*)	(Blankenship 1992; Frigaard and Dahl 2008; Hohmann-Marriott and Blankenship 2011; Madigan 2003)
Methylotrophs and methanotrophs	*Proteobacteria* (e.g., *Methylomonas*, *Methylocystis*, *Methylosinus*)	(Dunfield et al. 2003; Hanson and Hanson 1996; Semrau et al. 2008)
	Verrucomicrobia ("*Acidimethylosilex fumarolicum*", "*Methyloacida kamchatkensis*")	(Dunfield et al. 2007; Islam et al. 2008; Pol et al. 2007; Semrau et al. 2008)
Nitrogen fixation	*Actinobacteria* (e.g., *Frankia*, *Streptomyces*)	(Buckley et al. 2007; Huss-Danell 1997; Kevin Vessey et al. 2005; Pawlowski and Bisseling 1996; Ribbe et al. 1997; Wall 2000)
	Chlorobi (*Chlorobium*)	(Dos Santos et al. 2012; Farnelid et al. 2011)
	Chloroflexi (*Roseiflexus*)	(Dos Santos et al. 2012; Farnelid et al. 2011)
	Cyanobacteria (e.g., *Anabaena*, *Nostoc*)	(Gallon et al. 1991; Kevin Vessey et al. 2005; Newton 2007; Pawlowski and Bergman 2007; Rai et al. 2000)
	Firmicutes (e.g., *Bacillus*, *Clostridium*, *Paenibacillus*)	(Chen 2005; Dommelen and Vanderleyden 2007; Kennedy et al. 2004; Newton 2007)

Table 1.2 Continued

Functional activity	Phylum (examples of genera)	References
Nitrogen fixation	*Proteobacteria* (e.g., *Azoarcus*, *Azorhizobium*, *Azospirillum*, *Bradyrhizobium*, *Herbaspirillum*, *Pseudomonas*, *Rhodospirillum*, *Rhizobium*)	(Dommelen and Vanderleyden 2007; Falk et al. 1985; Kennedy et al. 2004; Kevin Vessey et al. 2005; Stacey 2007)
Aerobic ammonia/nitrite oxidation (Nitrification)	*Chloroflexi* ("*Nitrolancetus hollandicus*")	(Sorokin et al. 2012)
	Nitrospira (*Nitrospira*)	(Ehrich et al. 1995)
	Proteobacteria (e.g., *Nitrobacter*, *Nitrosomonas*, *Nitrosospira*)	(Head et al. 1993; Koops and Pommerening-Röser 2005; Koops et al. 1991; Sorokin et al. 1998)
Anaerobic ammonia oxidation	*Planctomycetes* (e.g., "*Anammoxoglobus*", "*Brocadia*", "*Jettenia*","*Kuenenia*", "*Scalindua*")	(Op den Camp et al. 2007; Strous et al. 1999; van de Graaf et al. 1996)
Denitrification	*Firmicutes* (e.g., *Bacillus*, *Geobacillus*)	(Liu et al. 2008; Suharti and de Vries 2005; van Spanning et al. 2007; Zumft 1997; Zumft and Körner 2007)
	Proteobacteria (e.g., *Azospirillum*, *Herbaspirillum*, *Pseudomonas*, *Rhodobacter*)	(Hiraishi et al. 1996; Ishii et al. 2011; Tago et al. 2011; van Spanning et al. 2007; Zumft 1997; Zumft and Körner 2007)
Dissimilatory nitrate reduction to ammonium	*Firmicutes* (e.g., *Clostridium*)	(Caskey and Tiedje 1979)
	Proteobacteria (e.g., *Desulfovibrio*, *Enterobacter*, *Pseudomonas*, *Wolinella*)	(Bokranz et al. 1983; Fazzolari et al. 1990; Samuelsson 1985; Seitz and Cypionka 1986; Strohm et al. 2007)

Table 1.2 Continued

Functional activity	Phylum (examples of genera)	References
Iron oxidizers	*Actinobacteria* (e.g., *Acidimicrobium*, *Ferrithrix*)	(Emerson et al. 2010; Johnson et al. 2009)
	Chlorobi (*Chlorobium*)	(Emerson et al. 2010; Hegler et al. 2008; Heising and Schink 1998)
	Firmicutes (*Alicyclobacillus*, *Sulfobacillus*)	(Emerson et al. 2010; Karavaiko et al. 2005; Norris et al. 1996)
	Nitrospira (*Leptospirillum*)	(Coram and Rawlings 2002; Emerson et al. 2010; Hippe 2000)
	Proteobacteria (e.g., *Acidithiobacillus*, *Leptothrix*, *Rhodobacter*, *Sideroxydans*)	(Corstjens et al. 1992; Emerson et al. 2010; Hallberg et al. 2010; Hegler et al. 2008)
Iron reducers	*Proteobacteria* (e.g., *Aeromonas*, *Desulfuromonas*, *Geobacter*, *Pseudomonas*, *Shewanella*, *Thiobacillus*)	(DiChristina et al. 2002; Knight and Blakemore 1998; Lovley et al. 1993; Nealson and Saffarini 1994; Straub et al. 1996)
Sulfur Oxidizers	*Chlorobi* (e.g., *Chlorobium*, *Chloroherpeton*)	(Frigaard and Dahl 2008; Ghosh and Dam 2009)
	Proteobacteria (e.g., *Acidithiobacillus*, *Rhodospirillum*)	(Frigaard and Dahl 2008; Ghosh and Dam 2009; Hallberg et al. 2010)
Sulfate and sulfur reducers	*Nitrospira* (*Thermodesulfovibrio*)	(Henry et al. 1994; Sekiguchi et al. 2008)
	Proteobacteria (e.g., *Desulfovibrio*, *Desulfobacter*, *Desulfuromonas*)	(Brandt and Ingvorsen 1997; Brysch et al. 1987; Schnell et al. 1989)

1.3.2 Assessing soil prokaryotic community structure and composition

1.3.2.1 Culture dependent and culture independent methods

Nowadays, soil microbial ecologists, in particular those studying prokaryotes, can use a wide variety of tools and techniques that permit the assessment of the microbial communities. All the techniques have associated drawbacks and benefits, and may extract different information about prokaryotic communities. Therefore, the method(s) chosen will depend on the objectives of the study.

The techniques may be divided into two main groups, those based on culture-dependent and those based on culture-independent methods. The major drawback of the former group is that only about 1 % of microorganisms (in particular, bacteria) are, so far, cultivable (Berg and Smalla 2009; Little et al. 2008; Scow et al. 2001). The inability to culture some microorganisms may rely on several factors such as, the requirement of specific growth conditions available in the natural environment (Davis et al. 2005; Davis et al. 2011), and/or the outcompetition by fast growing microorganisms (Davis et al. 2011; Scow et al. 2001; Shrestha et al. 2007). Other non-cultivability forms may correspond to viable but not in a cultivable state, for instance due to cell injuries (Kell et al. 1998; Liesack et al. 2000). Despite these limitations, culture-dependent methods are useful and quite informative. For instance, these methods, in particular the most probable number (MPN) (Weaver et al. 1994), allow the estimation of the abundance of microorganisms involved in certain metabolism (e.g., nitrogen fixation, autotrophy, heterotrophy, nitrate reducers) (Kidd et al. 2008; Nogales et al. 2002). The abundance of these microbial types may be a valuable indicator to compare soil samples from different locations or over a time scale. The fastness and low cost of these methods make them attractive, mainly when a first glance of the potential functional activities present in the belowground is aimed.

Culture-independent methods do not require any cultivation stage and are based on the examination of biochemical and molecular traits of organisms to characterize the taxonomic and functional activity of communities. Commonly used cellular

constituents are, for example, nucleic acids, lipids and proteins. Over the last years, most of the microbiota soil studies rely on nucleic acids. Although culture independent techniques were designed to get a full overview of each biological group in a sample, the complexity of some habitats and the limitations of the methods often hinder such objective. Soil is one of such habitats (Lombard et al. 2011; Young and Crawford 2004). The efficiency of the extraction of prokaryotic DNA is probably the major methodological limitation to get a full overview of the community, irrespective of the analysis method used (e.g., DGGE, T-RFLP, conventional cloning and sequencing techniques, high throughput sequencing techniques) (Delmont et al. 2011; Feinstein et al. 2009). Additional biases are associated with the PCR (e.g., preferential amplifications, primer efficiency and selectivity) (Derakshani et al. 2001; Röling and Head 2005; Scow et al. 2001). Despite the above mentioned and other drawbacks, the culture-independent methods revolutionized the prokaryote ecology studies revealing a so far unknown prokaryotic diversity (Janssen 2006; Rappé and Giovannoni 2003). Due to its use as gold standard in bacterial taxonomy and the availability of representative public databases, the 16S rRNA gene sequence analysis became a popular tool to characterize microbial communities in complex environments (Fierer et al. 2007b; Hartmann and Widmer 2006; Li et al. 2009; Olsen et al. 1986). DNA fingerprinting techniques (e.g., DGGE, T-RFLP) have been important tools for the comparison of the prokaryotic communities (Costa et al. 2007; Derakshani et al. 2001; Enwall et al. 2007). These fingerprinting methods may target different genomic regions (e.g., 16S rRNA or catabolic genes) (Junier et al. 2009; Krause et al. 2009; Sakurai et al. 2007; Stres et al. 2008) and give a fast overview of the structure of prokaryotic communities, being important for comparative purposes. DGGE is advantageous over T-RFLP because DGGE bands (DNA fragment) can be excised, cloned and sequenced supporting the identification of the corresponding organism (Gomes et al. 2005; Kikuchi et al. 2007), nevertheless these cloning procedure is time consuming and expensive. Fortunately, over the last years the nucleic acid sequencing technology had important technical advances. The development of high

throughput sequencing techniques, such as 454-pyrosequencing, among others (Mardis 2008; Suenaga 2012), which allow the analysis of millions of sequences at (comparatively to the Sanger method) reduced costs, contributed to increase the analysis output and promote bacterial community studies (Mardis 2008; Suenaga 2012). These techniques generate a high number of sequences per samples (ranging from several hundred thousand or from tens of millions of reads using 454-pyrosequencing and Illumina, respectively)(Mardis 2008), which allow a higher coverage of sample diversity than older sequencing techniques. Despite the bias associated (e.g., a large number of low-quality sequences, high computational requirements to analyse and process the huge amount of generated data)(Suenaga 2012; Zinger et al. 2012) the 454-pyrosequencing has been widely used in ecology studies (Baldrian et al. 2012; DeAngelis et al. 2011; Fierer et al. 2012), mainly because it generates sequences around 400 bp, which are more suitable for taxonomic affiliation than sequences around 100 bp generated by other high throughput sequencing techniques (Mardis 2011). For this reason, until now, most of the studies on soil bacterial diversity use 454-pyrosequencing approach.

1.3.2.2 Measurement of potential soil microbial activity

Soil functional activity may be inferred from the catabolic activity of the community, using community level physiological profiles (CLPP) (Kennedy 1994) or enzymatic assays (Weaver et al. 1994). In CLPP different organic substrates are tested individually as sources of carbon and energy for the members of the community. The final result is a pattern of substrates utilization. The enzymatic assays measure the activity of specific biocatalyst under controlled conditions, using known substrates for each enzyme family. Depending on the enzymatic activities tested, this type of assay may give an estimate of the intensity of biological activity or of the diversity of enzymes in the community (e.g., cellulase, phosphatase, arylsulfatase). CLPP and enzymatic patterning are not designed to identify the active members of a community, but to give a fast and low cost

snapshot of the potential functional activities of the soil. Both are useful tools to compare microbial communities.

1.3.3 Measuring microbial diversity and integration of environmental data

Over the years, microbiologists adopted and adapted some of animal and plants ecology concepts to meet the particular requirements of microbial ecology (Hughes et al. 2001). Currently, microbial ecology combines the most recent advances of the molecular biology with well consolidated approaches and concepts used in ecology, for example, to estimate and compare the richness and diversity of animals and plants in their communities.

1.3.3.1 Diversity measures

Indices that express the diversity and structure of a community are helpful tools to compare communities, mainly because they are very objective and easy to interpret. Two terms used to refer to biological communities are structure and diversity. By definition, structure refers to both the type and individual abundance of members that compose the community. Diversity refers to the number and individual abundance of each taxonomic or functional unit. Diversity can be measured over different ranges of biological organization, *i.e.*, from the gene to the phylum (Zinger et al. 2012). Because both measure different characteristics of the community, diversity and structure may vary independently (Little et al. 2008). Before assessing diversity it is crucial to define clearly the taxonomic or functional unit to be measured. Although this unit is frequently referred to as "species" it does not have necessarily the taxonomic meaning of species.

In general, in natural communities a few species are quite abundant while the majority of the others are minor representatives. There are several methods for measuring microbial diversity. Some methods are very simple consisting on the

establishment of a relationship between the number of different types of organisms observed *versus* the sampling effort (Hughes et al. 2001). These are designated as accumulation or a rank abundance curves. Examples of more sophisticated, statistic based methods, are the rarefaction (Heck Jr et al. 1975) and the richness estimators (Chao 1984). The rarefaction results from averaging randomizations of the observed accumulation curves, while richness estimators calculate the total richness of a sample by extrapolating beyond what has been recorded to estimate the unknown asymptote of a species accumulation curve, thus allowing comparisons across samples (e.g., nonparametric estimators, among others). The nonparametric estimators (e.g., Chao 1, abundance based coverage estimate - ACE) consider both the proportion of species that are not unique (*i.e.*, captured at least twice) and those that are observed only once. Given microbial communities have, in general, a high number of rare species and these estimators consider these low abundance classes, nonparametric estimators are the most promising estimation methods for microbial studies. Nevertheless, at low sample sizes both Chao 1 and ACE will underestimate the true richness. For this reason, analytical methods with higher outputs will offer higher accuracy on the estimates to be made. Therefore, estimating diversity based on 454-pyrosequencing data will be more accurate than based on cloning and sequencing data.

Advanced diversity indices use algorithms that combine species richness (*i.e.*, the number of species in a community) and evenness (measures how homogeneous is the abundance of a species in a community). This index varies between 0 and 1, respectively for totally uneven or totally even distribution of species in a community. The most commonly used diversity indices in ecology are the Shannon ($H = -\Sigma(n_i/N)\log(n_i/N)$, (Shannon and Weaver 1963), the Simpson (Simpson $=1-\Sigma(n_i/N)^2$;(Simpson 1949)), and the Evenness ($E = H/\log S$; (Pielou 1966)), where (S) corresponds to the number of species, (n_i) is the abundance of each species and (N) is the sum all individuals in the sample. The former index measures diversity while the others measure evenness. More recently, another diversity index, named phylogenetic diversity (PD), was developed (Faith 1992). This index is defined as

the minimum total length among all the phylogenetic branches required to embracing a given set of taxa on the phylogenetic tree. Thus, smaller PD values can be expected to correspond to smaller expected diversity.

Two other parameters (α and β diversity) are useful to compare the composition of microbial communities, mainly when large datasets are to be used. These parameters were defined by Whitthaker in 1972 and are still used. The α diversity refers to the number of species observed in a specific environment. The β diversity refers to the number of species shared between two different environments. A recently developed β diversity measure (UniFrac metric), incorporates also phylogenetic inference (Lozupone and Knight 2005). This upgrade of the β diversity measure takes into account the degree of divergence between related lineages, in addition to the presence or absence of species (unweighted Unifrac) or to the relative abundance of each species (weighted Unifrac).

1.3.3.2 Integration of ecological data

In general, in ecology studies several biotic and abiotic parameters are measured in the collected samples (habitat) being analysed, generating different datasets. Standard multivariate analysis, originally developed to integrate data from plant and animal ecology studies, are successfully used in microbial ecology studies (Noll et al. 2005; Sakurai et al. 2007). These methods measure the patterns of variation based on large species data sets (e.g., taxonomic or functional units, in terms of presence/ absence or abundance) and use correlation analyses to find significant relationships within the data set and between two data sets (e.g. abiotic and/or biotic environmental parameters and community composition). Within the same dataset, methods such as Principal Component Analysis (PCA) or Detrended Correspondence Analysis (DCA) are commonly used. The comparison of two datasets requires constrained multivariate analysis, using methods such as Canonical Correspondence Analysis (CCA) and Redundancy Analysis (RDA) (ter Braak 1994; ter Braak 1986).

1.4 Rice culture

More than 50 % of the world's population feeds on rice, making this one of the crops most produced worldwide (FAOSTAT 2010). This cereal is cultivated worldwide but the highest production is found in Asia (FAOSTAT 2010). Rice is produced in warm areas mainly under wetland conditions, although upland conditions can also be used. When cropped under wetland conditions, soil is flooded before rice planting, by seeding or seedling, and the flood conditions prevail over the rice cycle. Temporary drainage of the fields may occur when pesticides or fertilizers are applied (in conventional farming), or when soil is dried to avoid the growth of weeds (in organic farming). Rice growth under flooded conditions is advantageous because it brings the soil pH near to neutrality, increases nutrients availability (in particular, P and Fe), stimulates biological N_2-fixation, supplies nutrients from irrigation water, and prevents water percolation and soil erosion (Roger et al. 1993).

In Portugal, rice has been cultivated since the 18th century. Nowadays three major areas of rice production under wetland conditions are defined, at the basin of rivers Mondego, Tejo, and Sado (Direção Geral de Agricultua e DesenvolvimentoRural, Portugal, http://www.dgadr.pt/). Most of the fields are conventionally farmed, *i.e.*, mechanized production and utilization of synthetic fertilizers (N, P and K, using ammonium sulphate, ammonium dihydrogen phosphate and potassium chloride, respectively) and herbicides, such as propanil, MCPA, butachlor, benthazone and molinate. Until 2007, molinate was amongst the most used herbicides worldwide. Although still is use in several world regions (e.g., Europe) (http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection), molinate is no longer utilized in USA (http://iaspub.epa.gov/tdb/pages/contaminant/contaminantOverview.do?contaminantId=10620). In some small farms rice has been produced using traditional practices, without synthetic amendments or phytochemical protectors, and manual labour. Given the importance of rice cropping in Portugal, the "Direção Regional

de Agricultura e Pescas do Centro" (DRAPC) leads several field studies at the experimental farm "Bico da Barca" located in the valley of river Mondego (DRAPC, Portugal http://www.drapc.min-agricultura.pt/).

1.4.1 Paddy soils as special habitats

Rice (*Oryza sativa* L.) is an annual grass which growth can be divided into three agronomic stages of development: vegetative, reproductive, grain filling and maturation. The last stage, of grain filling and maturation, culminates with grain increase and colour change from green to gold, and the senescence of rice plant leaves. Most of these agronomic stages are related to changes occurring aboveground. Nevertheless, important changes occur also belowground during rice growth. For instance, at the early stage of rice growth, roots stimulate the growth of diazotrophs reaching the highest biological N_2-fixing activity close to the maximum tillering stage (Ikenaga et al. 2003; Knief et al. 2012; Sims and Dunigan 1984). Maximum tillering stage is preceded by a high root exudation activity (Ikenaga et al. 2003), which may stimulate the abundance of organotrophs and thus, of bacteria belonging to the phyla *Bacteroidetes*, *Firmicutes*, *Beta-* and *Gammaproteobacteria* abundant in the rice rhizosphere (Knief et al. 2012). The oxygen gradient in the rhizosphere may also shape the bacterial communities. Because young roots favor oxic environments and old roots favor anoxic environments, the gradient is directed from an oxic to an anoxic habitat and from the newest to the eldest roots. Therefore, differences may be also observed over the rice agronomic stages, with anaerobic bacteria being more abundant in the late agronomic stages than at the early stages (Ikenaga et al. 2003).

The fields where rice is produced, also known as paddy fields, are subjected to alternated periods of flooding and drainage. Such variations are associated with different states of soil water saturation, causing periodical changes in the redox potential, and making the paddy soil a unique agroecosystem. For instance, after flooding, oxygen is consumed rapidly due to aerobic bacteria and chemical

oxidation reactions, while the inorganic electron acceptors as nitrate, iron III, sulfate and carbon dioxide are reduced sequentially. At this stage, most of the soil becomes anoxic (Liesack et al. 2000; Roger et al. 1993). Nevertheless, even under flooded conditions some oxic compartments will still exist. Mainly in the rhizosphere, the diffusive transport of oxygen through the aerenchyma of rice roots allows the occurrence of various chemical and microbial oxidation processes (Schmidt et al. 2011). In paddy soils other important biological transformations take place, mainly under flooding conditions. For example, paddy soils are known for an intense denitrifying activity (Ishii et al. 2009), dissimilatory iron reduction (Treude et al. 2003), dissimilatory nitrate reduction to ammonium (Yin et al. 2002), and methanogenic activity (Watanabe et al. 2006; Watanabe et al. 2007). In addition, decomposing activity (e.g., rice straw and roots) is also an important microbial activity in paddy soils (Asari et al. 2007; Chin et al. 1999; Rui et al. 2009). The rice microbial food-web is driven by the contribution of the rice roots, which remain belowground after harvesting, and by rice straw, *i.e.*, the plant material remaining aboveground after harvesting, which, in general, is further ploughed in soil (Liesack et al. 2000; Rui et al. 2009). Therefore, an heterogeneous bacterial community is found in bulk soil, where aerobic and anaerobic bacteria affiliated to *Proteobacteria*, *Chloroflexi*, *Chlorobi*, *Verrucomicrobia*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, co-exist (Asakawa and Kimura 2008; Kikuchi et al. 2007; Xuan et al. 2012).

1.5 The main objectives of this study

Paddy soils have been the object of several scientific reports, which addressed the impressive microbial diversity that characterizes the belowground communities, and the potential emission of greenhouse gases by these soils. Nevertheless, there is still lack of knowledge on the effect of management practices on the paddy soil bacterial communities, though the importance of bacterial communities in the maintenance of soil fertility and on crop productivity is known. The scarce

information on the effect of rice production systems on the paddy soil bacterial communities combined with the need to find science-based methods to improve and promote sustainable rice production and to treat contaminated soils motivated this study. Thus, the present study aimed to assess i) the effect of different agriculture managements on the bacterial community of paddy soils; ii) the potential of some abiotic and biotic parameters to shape the bacterial communities in rice paddy fields; iii) and the feasibility of using bioremediation strategies to remediate contaminated paddy soils.

Chapter 2

Road map for the thesis

Rice is among the three most produced crops in the world, and its productivity has been boosted by conventional farming, *i.e*., addition of synthetic N fertilizers (in particular, urea) and herbicides (molinate, propanil, butachlor, among others). Beside all the environmental problems associated with pesticide and fertilizers use, possible impacts may be extensive to the composition and activity of soil microbiota. With access to farms using conventional and organic rice farming and given the scarcity of studies comparing microbial communities in soils using both management systems, a comparative study emerged as a good research opportunity. This comparison aimed to explore the functional and bacterial diversity of two paddy soils devoted to conventional and organic farming, respectively. The comparative study designed to assess the impact of conventional farming on the microbial community of bulk paddy soil is presented in Chapter 3.

The contamination of soil, leachate and receiving waters by molinate is well documented (Castro et al. 2005; Jiménez et al. 1999; Julli and Krassoi 1995; Park et al. 2005). This herbicide is one of the most recalcitrant thiocarbamates (Nagy et al. 1995) and toxic to different organisms of the trophic chain (Cochran et al. 1997; Galhano et al. 2009; Julli and Krassoi 1995). These facts combined with the observed changes in the bacterial community structure under conventional farming (Chapter 3) motivated further studies. At this stage, the need to develop a bioremediation process to attenuate the negative environmental impacts of molinate on contaminated paddy soils was evident. This objective was built also on the existence of a bacterial mixed culture (mixed culture DC) (Barreiros et al. 2003), which is, so far, the only culture able to mineralize molinate under a wide variety of operating conditions (Correia et al. 2006). The feasibility and potential for bioremediation of soils contaminated with molinate using either natural attenuation or bioaugmentation strategies was assessed, using microcosm assays. This study is described in Chapter 4.

Although it was possible to remove molinate from contaminated soil samples, irrespectively of the bioremediation strategies tested (Chapter 4), the avoidance of contamination is always preferred. According to farmers, the sustainable rice

production, *i.e.*, agriculture practices less dependent or completely independent of the use of synthetic compounds, is possible, and should be encouraged. Most of the organic managements adapt ancient agriculture methods, such as crop rotation systems. In the majority of the rotation systems, legumes rotate with a cereal. These systems are considered a good alternative to intensive farming (monoculture systems) due, not only, to their effective capacity to supply nitrogen to the following crop but also to suppress the growth of weeds, and, thus, maintain soil nutrients and crop yields. Nevertheless, the efficiency of these practices may be improved if the variations induced in the soil microbial activity and composition are understood. This information is a valuable tool to infer about microbe-microbe, microbe-plant or microbe- environment relationships. Therefore, the bacterial communities from two paddy soils at different stages of an alfalfa-rice rotation system were characterized using 454-pyrosequencing, as described in Chapter 5. In Chapter 6, 16S rRNA based metagenome analyses were integrated with all the biotic and abiotic data collected in these paddy soils, seeking for potential relationships between bacterial community structure and composition and external biotic or abiotic factors.

The results of this study offer an integrated perspective of the bacterial diversity and functional activity in bulk paddy soils under conventional *versus* organic farming, as well as under different stages of alfalfa-rice rotation system. Moreover, it demonstrates the metabolic diversity of paddy soil microbial communities. These topics are discussed in Chapter 7.

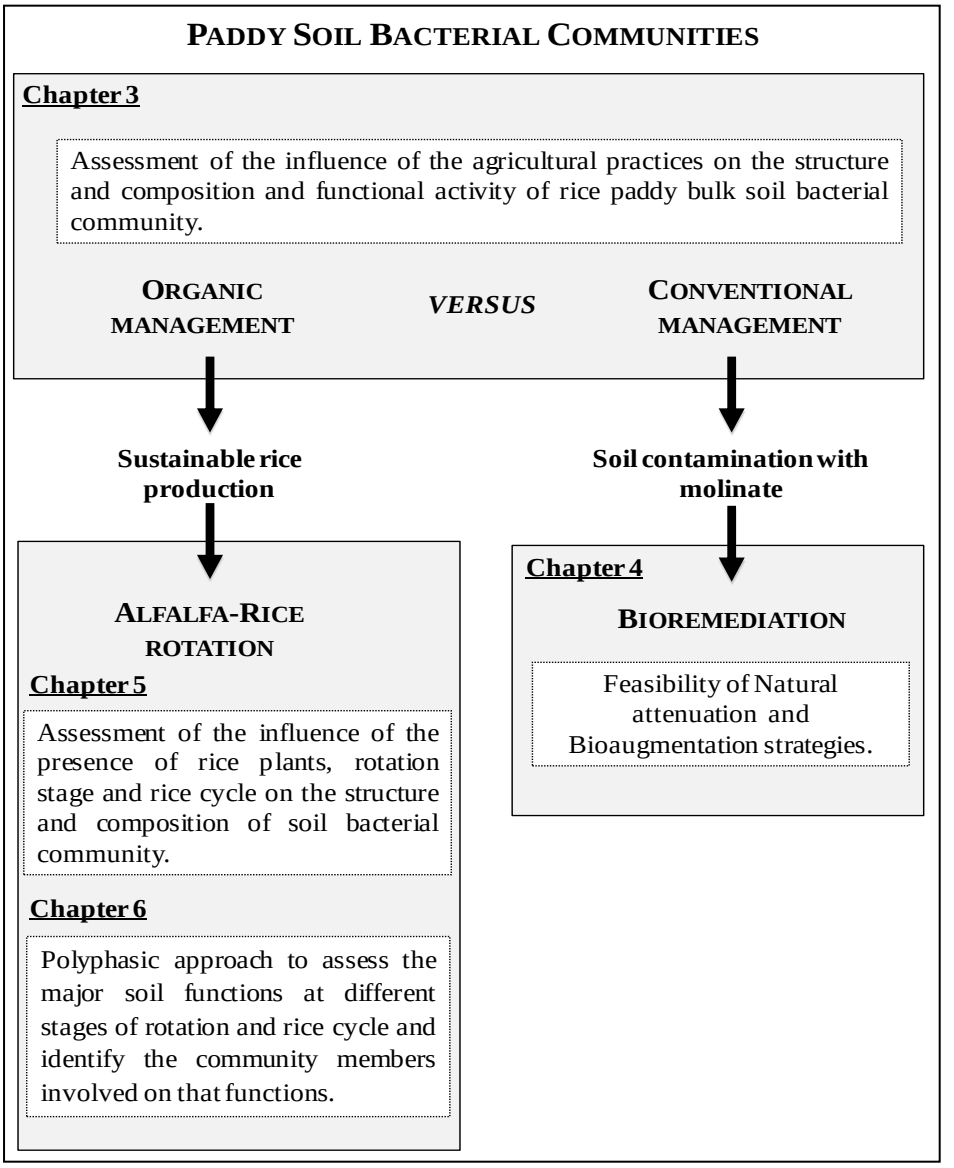

Fig. 2.1 Scheme of the major goals aimed in the present study.

Note: The core of this thesis is composed of four articles, two of which were published in peer-reviewed scientific journals (Chapters 3 and 4), the third (Chapter 5) and fourth (Chapter 6) are submitted for publication.

Chapter 3

Comparative study of the microbial diversity of bulk paddy soil of two rice fields subjected to organic and conventional farming

Results included in:

Lopes, A.R., Faria, C., Prieto-Fernández, A., Trasar-Cepeda, C., Manaia, C.M. and Nunes, O.C. (2011) Comparative study of the bacterial diversity of bulk paddy soil of two rice fields subjected to organic and conventional farming. Soil Biology and Biochemistry, 43: 115-125.

3.1 Abstract

Two adjacent paddies of an experimental rice field, subjected to organic and conventional farming, were characterized aiming the comparative assessment of microbiological variations occurring in the bulk paddy soil over the rice cycle. This study comprehended the simultaneous characterization of general physicochemical soil properties [total carbon and nitrogen, pH (H_2O and KCl), C:N ratio and water content], biochemical properties [enzymatic activities and Community Level Physiological Profiles (CLPP)], the estimation of cultivable organisms (enumeration of fast growing heterotrophic bacteria, actinomycetes and fungi) and the assessment of bacterial diversity using a culture-independent method (PCR-DGGE fingerprinting). The linkage of the parameters measured was analysed by canonical correspondence analysis (CCA).

CCA ordination plots of the CLPP showed a similar pattern of microbial functional activity in both agronomic management systems, except in June. Enzymatic activity, water content and fungi counts were the main factors affecting the observed CLPP time variation. Such a variation was not expressed by the Shannon and evenness indices, which did not evidence significant differences in the bacterial and functional diversity between or within farming type over the analysed period. The cluster and CCA analyses of the DGGE profiles allowed the distinction of the bacterial communities of both paddies, with temporal variations being observed in the organically managed field but not in the conventional paddy. Enzymatic activity, pH and molinate content were the factors which most contributed to the observed variations. Altogether these results underline the functional redundancy of the rice paddy soil and evidence the temporal variations on the metabolic activity of soil, irrespective of farming type.

Keywords: Cultivable microbial counts, soil enzymatic activity, CLPP, DGGE fingerprinting, Bacterial community diversity, Agronomic management.

3.2 Introduction

Soil is a complex ecosystem where living organisms play a key role in the maintenance of its properties. Soil biota comprises a huge diversity of organisms belonging to different taxonomic and physiologic groups, which interact at different levels within the community. Soil microorganisms constitute a source and sink for nutrients and are involved in numerous activities, such as transformation of C, N, P and S, degradation of xenobiotic organic compounds, formation of soil physical structure and enhancement of plants' nutrient uptake (Gregorich et al. 1994; Seklemova et al. 2001). For these reasons, the importance of microorganisms in the maintenance of quality and productivity of agricultural soils is unquestionable. The responsiveness of microorganisms to environmental factors implies that disturbances imposed by agricultural treatments may lead to alterations in the composition and activity of soil microbiota and, therefore, may affect soil quality (Gregorich et al. 1994; Shibahara and Inubushi 1997).

In the last decades, conventional management of agricultural soils, namely tillage and the utilization of synthetic fertilizers and plant protectors (pesticides), has been implemented worldwide, to improve the productivity and the quality of agricultural goods at low cost (Hasset and Banwart 1992). Nevertheless, the recognition that intensive conventional farming promotes soil degradation (Liu et al. 2007; Mäder et al. 2002), erosion (Eltun et al. 2002), and environmental contamination (e.g. Castro et al., 2005) led to a widespread interest in organic farming, with the natural control of pests and the utilization of compost and/or manure to substitute synthetic fertilizers (Mäder et al. 2002; Shibahara and Inubushi 1997). Over the last years, the effect of agricultural management practices on the soil properties and on crop yield has been widely studied in diverse types of agricultural soils (Liu et al. 2007; Mäder et al. 2002; McCaig et al. 2001).

Rice paddy soil is considered a unique agro-ecosystem as it is kept flooded during the rice growth and is drained during the off crop season. The rice paddy field comprises three compartments where the soil is subjected to different physical and

chemical conditions: the oxic surface, the anoxic bulk and the rhizosphere plus rhizoplane (Liesack et al. 2000). Thus, the high diversity of habitats in the paddy field ecosystem offers a spatial and temporal heterogeneity, which enhances prokaryote specialization and division into distinct ecological niches (Henckel et al. 2001). Most of the microbiological studies in this type of ecosystem have been focused on the effect of oxygen concentration in the different field compartments, and on specific processes, particularly, methane emission and consumption, N2 fixation, N mineralization and sulphate reduction (Henckel et al. 2001; Liesack et al. 2000; Roger and Ladha 1992; Shibahara and Inubushi 1997; Wind and Conrad 1997). Different fertilization regimes and the incorporation of organic residues are the most commonly addressed issues in studies focusing on agricultural management of paddy fields (Shibahara and Inubushi 1997; Watanabe et al. 2006; 2007; Zhong and Cai 2007). Nevertheless, comparative studies between conventional management and organic farming are scarce, if available, for rice fields. The worldwide extension and importance of this crop and the availability of an experimental rice field where such a comparison was being made, having in mind the assessment of productivity yields, made possible and motivated the current study.

In the present work, bulk soil from two adjacent paddies of an experimental rice field subjected to different management (organic and conventional) was compared. Our major goals were the comparison of the effect of the agricultural management on soil properties and the assessment of temporal variations. Specifically we intended to determine if the functional and bacterial diversity of soil varied over time and if such variations were similar irrespectively of the farming type. With this objective, soil subjected to different managements was sampled simultaneously at four different times over the rice cycle and was characterized using different approaches. The analyses carried out included the soil general characterization as well as measurements of biochemical properties, counts of total cell numbers and cultivable organisms and the assessment of bacterial diversity using a culture-independent method.

3.3 Materials and methods

3.3.1 Experimental field

The study was conducted in two adjacent paddy fields, each with three replicate plots, named I, II and III, from the experimental farm "Bico da Barca", from Direção Regional de Agricultura e Pescas do Centro (DRAP C), located in the valley of river Mondego, Montemor-o-Velho, Central Portugal (40º11 'N; 08º41 'W). These fields have the same soil type and cropping system, but have received different soil management practices, organic (ORG) and conventional (CONV). Paddy field ORG (4200 m^2) was under organic production for two years (2005-2007), and before it was uncultivated. Paddy field CONV (7100 m^2) was subjected to conventional management for six consecutive years.

Rice was seeded in both fields in spring 2007 (Fig. 3.1). The field management of paddy ORG was as follows: basal fertilization with mineral gafsa (Fertigafsa, 300 kg P ha^{-1}) and organic amendment with a plant residue commercial compost (Fertiormont, 2 t ha^{-1}) in early April; flooding on 16th April; drainage and mechanical removal of weeds on 20th May; flooding on 21st May; seeding on 22nd May. The field management of paddy CONV was as follows: flooding on 19th April; seeding on 23rd April; molinate (Ordram, 50 kg ha^{-1}) application on 26th April; drainage on 1st June; propanil (Stam, 18 L ha^{-1}) and bentazone (Basagram, 4 L ha^{-1}) application on 4th June; flooding on 6th June; and fertilization (102 kg N, 63 kg P_2O_5 and 35 kg K_2O per hectare, as ammonium sulphate, ammonium dihydrogen phosphate and diammonium monohydrogen phosphate, and potassium chloride, respectively) on 8th June. Both paddies ORG and CONV were drained on 18th September, and harvested between 18th and 21st September.

3.3.2 Soil sampling

In both paddy fields, triplicate composite samples (each consisting of three different soil cores pooled together) were collected from the upper 0-15 cm of the

soil from each of the three plots (I, II and III). The area of each plot of paddies ORG and CONV was of 1400 and 2367 m^2, respectively. In order to assess possible modifications caused by the main agronomic management activities in the fields during the rice cycle, soil samples were collected on four occasions - 23rd April, 2nd May,13th June and 15th October of 2007. Soil samples were homogenized and cleaned by manual mixing of soil cores and removal of visible root debris, respectively. Soil samples were immediately processed for microbiological characterization or stored at 4 ºC (for no more than 2 weeks) and -20 ºC, before processing for biochemical and molecular characterization, respectively.

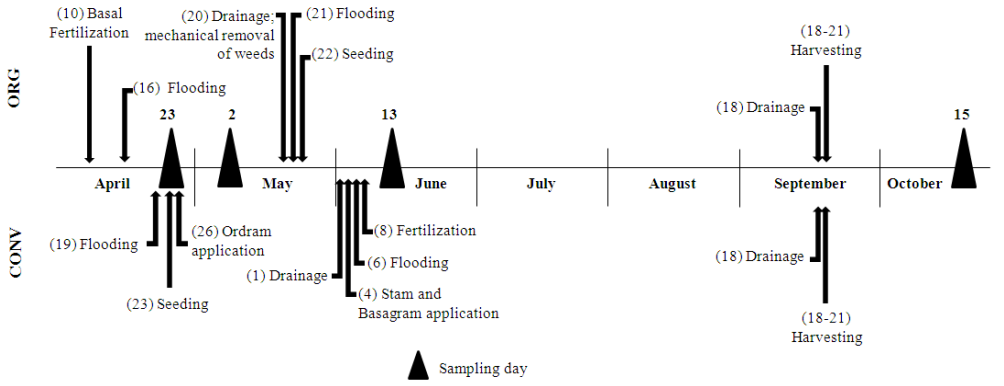

Fig. 3.1 Agricultural management procedures of paddy fields ORG and CONV and sampling dates.

3.3.3 General soil characterization

The physical and the chemical properties of bulk soil were analysed following the procedures described by Guitián and Carballas (1976). Briefly, total organic C was determined by wet oxidation with potassium dichromate, after treatment with H_2SO_4 to facilitate digestion of the organic matter and to ensure the removal of any inorganic C present; total N was measured by the Kjeldahl digestion method and the pH in water and in 1 M KCl was determined in a soil:solution mixture (1:2.5

w:v), with a glass electrode. Molinate concentration in bulk soil was determined by gas-chromatography-mass spectrometry, after soxhlet extraction with dichloromethane in Agência Portuguesa do Ambiente (http://www.apambiente.pt).

3.3.4 Enumeration of total cells and of cultivable microorganisms

The enumeration of total cells from bulk soil was performed by the 4,6-diamidino-2-phenylindole (DAPI) staining method as described by Brunk et al. (1979). For each sample 10 g of soil were suspended in 90 ml of sterile saline solution (0.85 % NaCl, w/v) and the mixture was stirred for 30 min at 200 rpm, sonicated (sonication bath) for 5 min, and centrifuged at 3000 rpm for 5 min. The enumeration of total cells was performed in the supernatant, as described by Manuel et al. (2007).

Fast growing cultivable microorganisms were enumerated using the membrane filtration method. Serial dilutions of the soil suspensions were filtered through a 47 mm membrane with 0.45 µm porosity (Knowles and Barraquio 1994). The media Plate Count Agar (Merck), Actinomycetes Isolation Agar (Merck) and Rose Bengal-Chloramphenicol (Merck) were used, respectively, for the enumeration of fast growing heterotrophic bacteria, actinomycetes and fungi. Plate Count Agar and Actinomycetes Isolation Agar cultures were incubated at 30 ºC for 48 h and 7 d, respectively; Rose Bengal-Chloramphenicol cultures were incubated at 22 ºC for 7 d. Data from triplicates were expressed as colony forming units (CFU) g^{-1} dry soil (oven-dried soil basis).

3.3.5 Soil enzymatic activities

Dehydrogenase activity was determined with iodonitrotetrazolium violet (INT) as substrate, incubating with 1 M TRIS-HCl buffer pH 7.5 for 1 h. The iodonitrotetrazolium formazan (INTF) produced was extracted with a 1:1 (v:v) mixture of ethanol and dimethylformamide and measured spectrophotometrically at

490 nm. Activity was quantified by reference to a calibration curve constructed using INTF standards incubated with soil under the same conditions described above, and is expressed in mmol INTF g^{-1} h^{-1} (Camina et al. 1998). Catalase activity was determined according to the Trasar-Cepeda et al. (1999) modification of the method of Johnson and Temple (1964). The soil samples were incubated with H_2O_2 for 10 min and the residual H_2O_2 was determined by a colorimetric method. The activity is expressed in mmoles H_2O_2 g^{-1} h^{-1}.

The activity of arylsulphatase was measured by using the method of Tabatabai and Bremner (1970), with minor modifications. Briefly, arylsulphatase activity was determined with 5 mM *p*-nitrophenyl sulphate as substrate, with 0.5 M acetate buffer (pH 5.8). After incubating for 1 h at 37 ºC, 2 M $CaCl_2$ was added and the liberated *p*-nitrophenol was extracted with 0.2 M NaOH. The enzymatic activity was quantified by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each soil under the same conditions as for the samples and the activity is expressed as µmol *p*-nitrophenol g^{-1} h^{-1}. The activity of urease was determined as described by Nannipieri et al. (1980). Briefly, urease activity was determined using 1065.6 mM urea as substrate, incubating for 1.5 h in 0.2 M phosphate buffer (pH 8.0), and measuring the NH_4^+ released with an ammonia electrode, and the enzyme activity is expressed as µmol NH_3 g^{-1} h^{-1}. The activity of protease hydrolysing casein (casein-protease) was determined with 1 % casein as substrate, incubating for 2 h in 0.05 M Tris(hydroxymethyl)aminomethane-HCl (TRIS-HCl) buffer (pH 9.0) and the released amino acids determined by the Folin-Ciocalteu colorimetric method described by Ladd and Butler (1972), modified by Nannipieri et al. (1979). The casein-protease activity is expressed as µmol tyrosine g^{-1} h^{-1}. Invertase activity was determined with 35.06 mM saccharose as substrate, incubating for 3 h, with 2 M acetate buffer (pH 5.5), the released reducing sugars determined following the method of Schinner and von Mersi (1990) and the enzymatic activity expressed as µmol glucose g^{-1} h^{-1}.

All determinations were performed in triplicate and the average activity values were expressed on an oven-dried soil basis.

3.3.6 Soil community level physiological profiles (CLPP) analysis

The ability of microbial communities to utilize different C substrates was determined using the method of Kennedy (1994). Five grams of each bulk soil replicate sample were suspended in 45 ml of 1 % sodium hexametaphosphate and shaken for 30 min, vortexed at maximum speed for 1 min and serially diluted (1:10). Fifty microliter aliquots of the dilutions 10^{-2}-10^{-7} were inoculated in 96-well plates containing different C sources (amines: β-phenyl ethylamine and putrescine; amino acids: $_{L}$-arginine, $_{L}$-asparagine; $_{L}$-glutamic acid, $_{L}$-phenylalanine, $_{L}$-serine, $_{L}$-threonine; carbohydrates: $_{D}$-(+) cellobiose, α-lactose, β-methyl $_{D}$-glucoside, $_{D}$-(+) xylose, i-erythritol, maltose, *N*-acetyl-$_{D}$-glucosamine, glyceraldehyde; carboxylic acids: $_{D}$-galactonic acid γ-lactone, galacturonic acid, o-hydroxybenzoic acid, p-hydroxybenzoic acid, malonic acid, α-keto butyric acid, malic acid; polymers: Tween 40, Tween 60, α-cyclodextrin, glycogen and miscellaneous: α-$_{D}$-glucose-1-phosphate) and prepared as described by Kidd et al. (2008). Substrate utilization was indicated by colour development of the tetrazolium violet redox dye (2,5-diphenyl-3-(α-naphthyl)tetrazolium chloride (TV), 0.15 mM) after 7 d of aerobic incubation at 25 ºC. The total number of C sources utilized was recorded and the number (MPN) of cells grown at each substrate was estimated using tables for 3 replicates per dilution level.

3.3.7 Bacterial community analysis

Bulk soil DNA was extracted from 0.25 g of soil using Power-Soil™ DNA Isolation Kit (MO BIO) with an additional incubation step at 65 ºC for 15 min, after 40 min agitation at 1300 rpm. Quality of extracted DNA was controlled in 1 % agarose gels.

For PCR-DGGE profiling, a 500 bp fragment (based on the reference strain *Escherichia coli* bases 984 and 1378) was amplified using the 16S rRNA gene primers forward F984GC, containing a GC clamp (50-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-30) and reverse R1378 (50- CGG TGT GTA CAA GGC CCG GGA ACG-30) (Heuer et al., 1997, clamp Nübel et al., 1996). The reaction mixtures (50 µl) contained 2 µl of target DNA, 0.5x PCR buffer with 50 mM KCl, 0.5x PCR buffer with 20 mM $(NH_4)_2SO_4$, 0.4 mM dNTP's, 3 mM $MgCl_2$, 5 % DMSO, 0.6 µM of each primer, and 3 U taq DNA polymerase (Fermentas). Thermal cycling conditions were as follows: 5 min at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 64 ºC and 1 min at 72 ºC and a final extension step of 30 min at 72 ºC. Amplified products were quantified in Qubit® Fluorometer (Invitrogen) with Quant-iT™ dsDNA HS assay kit.

Amplified bacterial 16S rRNA gene fragments (~75 ng of DNA) were separated in a double gradient polyacrylamide gel containing 6-9 % acrylamide, to improve band resolution (Cremonesi et al. 1997) and a gradient of 30-58 % of denaturant (100 % denaturant corresponds to 7 M urea and 40 % (v/v) formamide), using D-code System (Bio-Rad). The electrophoresis run in 1x TAE buffer at 60 ºC at a constant voltage of 200 V for 6 h and was preceded by a pre run at 60 V for 30 min. The DGGE gels were silver-stained according to Heuer et al. (1997).

3.3.8 Statistical analyses

Scanned DGGE gels were analysed with the Bionumerics software (version 6.0, Applied Maths, Belgium). DGGE gels were normalized using a standard reference sample, which was loaded in 3 lanes in every gel. Samples were compared after band-matching analysis in the area 19.3-84.2 % of the gel. Bands were assigned to classes of common bands within all profiles, and band-matching tables based on densitometric values were obtained. These band-matching tables were the basis for community cluster and ordination analysis and for the determination of diversity

indices. Similarity between fingerprints was calculated using the Pearson product moment correlation coefficient and dendrograms were generated using the unweighted pair-group method with arithmetic averages (UPGMA).

Canonical Correspondence Analysis (CCA) was carried out in order to assess the influence of general soil properties, biochemical and microbiological parameters (explanatory variables) on the variance of the bacterial community composition (DGGE patterns) or microbial functional activity (growth on single C source), within and between the soil of paddies ORG (paddy ORG) and CONV (paddy CONV), over the rice cycle. CCA was performed using PCORD (version 5, MJM Software, Gleneden, USA). The significance of the relationship between community data (principal matrix) and the environmental data (second matrix) was tested by Monte Carlo permutations test (n = 999).

Explanatory variables included in both CCA analyses were selected by manual forward selection including the permutation test (Monte Carlo permutations test). All the soil properties and biochemical and microbiological parameters for which the null hypothesis was excluded ($P < 0.05$) were included in the ordination.

Abiotic parameters, microbial counts, enzymatic activity and diversity indices within each paddy were compared using a two way analysis of variance (ANOVA) in function of the sampling time. The post-hoc Tukey test was applied when adequate. Paddies ORG and CONV were also compared using the two-sample statistical test (t-test). The statistical analyses were done using Excel software package (Microsoft Excel).

3.3.9 Analysis of soil functional and structural diversity

The structural and functional diversity of the microbial community was assessed using the Shannon index of diversity ($H = - \Sigma(n_i/N)\log(n_i/N)$; (Shannon and Weaver 1963)) and the evenness index ($E = H/\log S$; (Pielou, 1966)). For the bacterial community analysis, the number of DGGE bands corresponded to the number of species (S) in each sample while for the functional analysis, the number of

substrates metabolised (*S*) in CLPP analysis corresponded to the number of carbon sources used for growth by each sample. The relative surface intensity of each DGGE band or the log MPN g^{-1}dry soil value associated with each substrate (n_i) and the sum of all the surface band intensity or of all the log MPN g^{-1}dry soil values associated with substrates used in a given sample (*N*) were used, respectively, as estimates of species abundance or extent of its use (Fromin et al. 2002).

3.4 Results

3.4.1 General soil characterization

Among the physicochemical parameters analysed in the paddies ORG and CONV, Water content varied significantly throughout the rice cycle ($P < 0.01$) (Table 3.1). As could be anticipated, the average water content values were lower in October, when the two paddies were drained. In spite of some minor differences, paddies ORG and CONV differed significantly only for pH, in April and for the water content, in May and June. In April, pH H_2O and pH KCl values were higher in paddy ORG than in CONV ($P < 0.05$). In comparison with paddy CONV, paddy ORG water content was higher in May and lower in June ($P < 0.05$). As expected, molinate was detected only in paddy CONV; the highest concentration was found immediately after its application, being also detected about one month later.

Table 3.1 Main physical and chemical properties of soil of paddies ORG and CONV. Values are means ± standard deviation (n = 3).

Parameter	April		May		June		October	
	ORG	CONV	ORG	CONV	ORG	CONV	ORG	CONV
pH in water	6.44±0.17 ^{A*}	5.67±0.35 ^{A*}	6.32±0.05 ^{A}	6.21±0.09 ^{A}	6.33±0.15 ^{A}	6.00±0.27 ^{A}	6.23±0.02 ^{A}	6.03±0.29 ^{A}
pH KCl	4.78±0.09 ^{A*}	4.34±0.25^{A*}	5.01±0.21^{A}	4.76±0.20 ^{A}	4.92±0.03^{A}	4.78±0.33^{A}	4.88±0.06 ^{A}	4.49±0.31 ^{A}
Total C (%)	1.91±0.13 ^{A}	1.94±0.24 ^{A}	1.83±0.19 ^{A}	2.14±0.11^{A}	1.76±0.45 ^{A}	2.25±0.36 ^{A}	1.43±0.14 ^{A}	1.74±0.35 ^{A}
Total N (%)	0.17±0.02 ^{A}	0.18±0.01 ^{A}	0.18±0.02 ^{A}	0.19±0.01^{A}	0.16±0.01 ^{A}	0.17±0.02 ^{A}	0.16±0.01 ^{A}	0.16±0.01 ^{A}
C/N	12±1 ^{A}	11±1 ^{A}	10±0^{A}	11±1^{A}	11±3 ^{A}	14±1 ^{A}	9±1 ^{A}	11±3 ^{A}
Water content (%) (g H_2O/100 g wet soil)	35.9±0.6 ^{C}	36.4±0.8 ^{B}	35.7±0.3 ^{C}*	34.1±0.9 ^{B}*	28.2±0.7 ^{B}*	37.6±2.3 ^{B}*	24.7±1.1 ^{A}	25.0±0.9 ^{A}
Molinate (mg kg^{-1})	< 0.050 ^{A}	< 0.050 ^{A}	< 0.050 ^{A*}	0.297±0.005 ^{C*}	< 0.050 ^{A*}	0.163±0.003 ^{B*}	< 0.050 ^{A}	< 0.050 ^{A}

The texture of soil of paddies ORG and CONV was, respectively, sand: 28.2 and 28.2%; silt: 51.1 and 49.7%; and clay: 20.7 and 22.1%. The total exchange bases and the cation exchange capacity of soil of paddies ORG and CONV were, respectively, 9.3 and 6.6 cmol(C) kg^{-1}, and 9.3 and 10.0 cmol(C) kg^{-1}; These analyses were determined in Instituto Nacional de Recursos Biológicos, Departamento de Ciência do Solo da Estação Agronómica Nacional, Oeiras, Portugal. Molinate limit of quantification was 0.05 mg kg^{-1}. A-C, Homogeneous subsets within each paddy (ORG or CONV), as determined by the Tukey test at $P < 0.05$. *, Significant differences between paddies ORG and CONV on basis of the two-sample *t*-test at $P < 0.05$.

3.4.2 Total and cultivable microorganisms

In paddy ORG the number of total cells did not vary significantly over the rice cycle (Table 3.2). In contrast, cultivable microorganisms varied significantly throughout the period under study. In June the number of heterotrophs was higher than in April ($P < 0.05$) but was not different from those of May or October. The number of actinomycetes reached its maximum in June ($P < 0.05$) although not significantly different from October. The number of fungi was also higher in June and October ($P < 0.05$) than in April and May.

In paddy CONV the total number of cells was higher in June and October than in April and May ($P < 0.05$). Among the cultivable populations, only the number of heterotrophs varied significantly over the rice cycle, with higher counts in June than in April and May ($P < 0.05$), but not significantly different from those observed in October.

When comparing both paddies, the only significant difference was observed in June, when the total number of cells was slightly higher for paddy CONV than for paddy ORG ($P < 0.05$).

Table 3.2 Enumeration of heterotrophic bacteria, actinomycetes and fungi in soil of paddies ORG and CONV over the rice cycle. Values are means ± standard deviation (n = 3).

	Total cells		Heterotrophs		Actinomycetes		Fungi	
	log Cells g^{-1}dry soil		log CFU g^{-1} dry soil					
	ORG	CONV	ORG	CONV	ORG	CONV	ORG	CONV
April	8.0 ± 0.2 ^{A}	7.9 ± 0.1 ^{A}	5.0 ± 0.6 ^{A}	5.6 ± 0.4 ^{A}	5.2 ± 0.3 ^{A}	5.5 ± 0.6 ^{A}	3.4 ± 0.3 ^{A}	3.7 ± 0.3 ^{A}
May	7.9 ± 0.2 ^{A}	7.8 ± 0.1 ^{A}	5.7 ± 0.2 ^{A,B}	5.5 ± 0.3 ^{A}	5.3 ± 0.4 ^{A}	5.4 ± 0.6 ^{A}	3.2 ± 0.1 ^{A}	3.4 ± 0.6 ^{A}
June	7.7 ± 0.1 ^{A*}	8.1 ± 0.1 ^{B*}	6.4 ± 0.2 ^{B}	6.4 ± 0.3 ^{B}	6.3 ± 0.3 ^{B}	6.1 ± 0.3 ^{A}	4.6 ± 0.1 ^{B}	4.2 ± 0.4 ^{A}
October	8.1 ± 0.1 ^{A}	8.2 ± 0.1 ^{B}	5.8 ± 0.2 ^{A,B}	5.9 ± 0.2 ^{A,B}	5.6 ± 0.3 ^{A,B}	5.6 ± 0.3 ^{A}	4.3 ± 0.4 ^{B}	4.3 ± 0.3 ^{A}

A-B, Homogeneous subsets within each paddy (ORG or CONV), as determined by the Tukey test at $P<0.05$.

*, Significant differences between paddies ORG and CONV on basis of the two-sample *t*-test at $P<0.05$.

3.4.3 Soil enzymatic activities

Four out of the six enzymatic activities analysed presented significant temporal variations both in ORG and CONV paddies. Urease and catalase activities presented significant variations in both paddies, with the highest value in May. Activity of invertase showed similar variations in both paddies though changes were only significant for paddy ORG; the highest value was registered in April, although not significantly different from that observed in May and October. Dehydrogenase activity varied in paddy CONV, with its maximum in June (Table 3.3).

When paddies ORG and CONV were compared for their patterns of enzymatic activity it was observed that in April paddy CONV showed a higher urease activity than paddy ORG ($P < 0.05$). In May, the dehydrogenase activity was higher in paddy ORG than in paddy CONV ($P < 0.05$) and in October, arylsulphatase activity was higher in paddy CONV than in paddy ORG ($P < 0.05$).

Table 3.3 Enzymatic activity of soil of paddies ORG and CONV over the rice cycle. Values are means ± standard deviation (n =3).

	Dehydrogenase (μmol INTF g^{-1} h^{-1})		Catalase (mmol H_2O_2 g^{-1} h^{-1})		Invertase (μmol glucose g^{-1} h^{-1})	
	ORG	CONV	ORG	CONV	ORG	CONV
April	0.28±0.1 ^{A}	0.21±0.03 ^{A}	0.46±0.17 ^{A}	0.28±0.13 ^{A}	2.58±0.29 ^{B}	2.37±0.71 ^{A}
May	0.51±0.09 ^{A*}	0.23±0.02 ^{A*}	1.51±0.14 ^{B}	1.39±0.06 ^{C}	1.93±0.23 ^{A,B}	1.63±0.43 ^{A}
June	0.20±0.02 ^{A}	0.57±0.33 ^{B}	1.32±0.09 ^{B}	1.11±0.18 ^{C}	1.80±0.36 ^{A}	1.81±0.52 ^{A}
October	0.46±0.21 ^{A}	0.18±0.03 ^{A}	0.68±0.16 ^{A}	0.66±0.14 ^{B}	2.27±0.15 ^{A,B}	2.35±0.02 ^{A}

Table 3.3 Continued

	Urease (μmol NH_3 g^{-1} h^{-1})		Casein-protease (μmol tyrosine g^{-1} h^{-1})		Arylsulphatase (μmol PNPg^{-1} h^{-1})	
	ORG	CONV	ORG	CONV	ORG	CONV
April	0.87±0.09 ^{A*}	1.14±0.05 ^{A*}	0.41±0.09 ^{A}	0.36±0.09 ^{A}	0.09±0.01 ^{A}	0.11±0.01 ^{A}
May	2.08±0.29 ^{C}	2.51±0.58 ^{B}	0.43±0.01 ^{A}	0.43±0.06 ^{A}	0.13±0.03 ^{A}	0.13±0.02 ^{A}
June	1.33±0.19 ^{A,B}	1.55±0.74 ^{A,B}	0.37±0.11 ^{A}	0.40±0.10 ^{A}	0.11±0.02 ^{A}	0.15±0.02 ^{A}
October	1.61±0.33 ^{B,C}	1.62±0.24 ^{A,B}	0.25±0.09 ^{A}	0.29±0.09 ^{A}	0.10±0.01 ^{A*}	0.14±0.01 ^{A*}

A-C, Homogeneous subsets within each paddy (ORG or CONV), as determined by the Tukey test at $P<0.05$.

*, Significant differences between paddies ORG and CONV on basis of the two-sample *t*-test at $P<0.05$.

3.4.4 Soil community level physiological profiles (CLPP) analysis

All substrates analysed supported the growth of the microbial communities of both paddies and in every sampling time studied (Fig. 3.2). The average values of Shannon index (*H*) and of evenness index (*E*) for functional diversity in both paddies were respectively of 1.44 and of 1.00. No significant differences between paddies or over time were observed for these indices (Table 3.4). The highest MPN of microorganisms growing on the 21 out of the 28 analysed substrates was registered in June for both paddies (Fig. 3.2). The exceptions were maltose, putrescine, o-hydroxybenzoic and p-hydroxybenzoic acid, $_{D}$-(+)xylose, $_{D}$-(+)cellobiose, $_{L}$-asparagine. The lowest values were observed in October, except for glycogen, L-serine, a-lactose, galacturonic acid in paddy ORG and α-keto butyric acid, β-methyl $_{D}$-glucoside, $_{L}$-glutamic acid, $_{D}$-galactonic acid γ-lactone in paddy CONV. In the CCA biplot, the total variance in the CLPP data explained by axis 1 and 2 was 73.4% (53.8 and 19.6%, respectively) (Fig. 3.3). The species-environmental correlations were high, with values of 0.999 and 0.967 for axis 1 and 2, respectively ($P < 0.001$). The temporal variation of the community functional activity of both soils was explained mostly by the urease activity, the fungi counts, which presented high intraset correlations with axis 1 (-0.791 and 0.715, respectively) and the catalase activity and soil water content, which presented higher intraset correlations with axis 2 (-0.730 and 0.591, respectively). Except in June, paddies ORG and CONV showed a similar pattern of microbial functional diversity temporal variation. Malonic (-0.615, $P < 0.001$) and o-hydroxybenzoic (-0.517, $P < 0.001$) acids, for which higher MPN values were registered in May than in October in both paddies, were among the substrates that most contributed to the variation observed along axis 1. Glycogen (-0.796, $P < 0.001$), $_{L}$-asparagine (-0.720, $P < 0.001$), and putrescine (-0.711, $P < 0.001$) were the substrates that most contributed to the variation observed along axis 2. For these substrates, the lowest MPN values were registered in April in both paddies, whereas in June, higher MPN values were observed in paddy ORG than in paddy CONV.

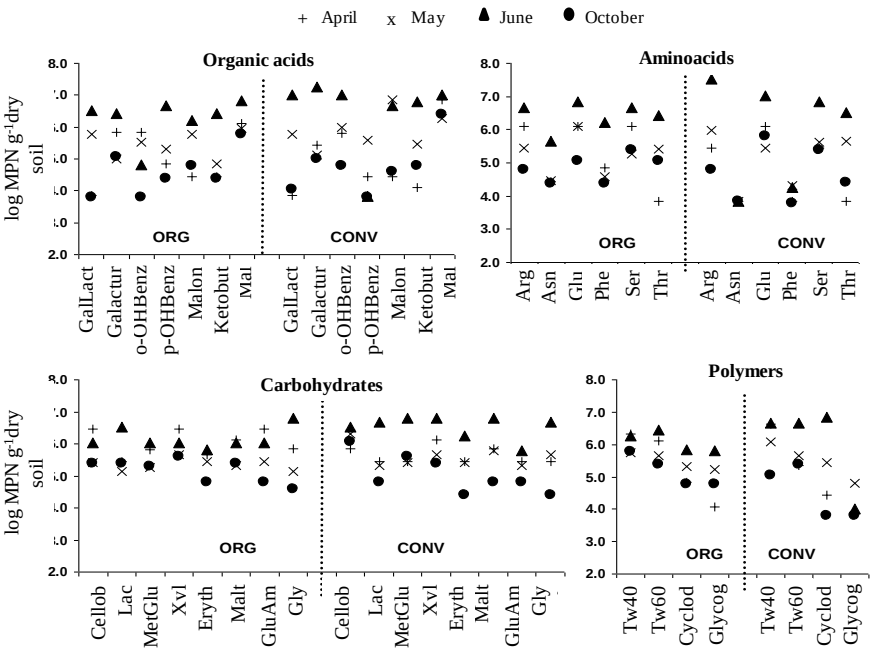

Fig. 3.2 Density of microorganisms in soil from paddies ORG and CONV growing in some of the tested substrates, over the rice cycle.

GalLact: $_D$-galactonic acid δ-lactone; Galactur: galacturonic acid; o-OHBenz: o-hydroxybenzoic acid; p-OHBenz: p-hydroxybenzoic acid; Malon: malonic acid; Ketobut: α-keto butyric acid; Mal: malic acid. Arg: $_L$-arginine; Asn: $_L$-asparagine; Glu: $_L$-glutamic acid; Phe: $_L$-phenylalanine; Ser: $_L$-serine; Thr: $_L$-threonine. Cellob: $_D$-(+) cellobiose; Lac: α-lactose, MetGlu: β-methyl D-glucoside; Xyl: $_D$-(+) xylose, Eryth: i-erythritol, Malt: maltose; GluAm: *N*-acetyl-$_D$-glucosamine, Gly: glyceraldehyde. TW40: Tween 40; Tw60: Tween 60; Cyclod: α-cyclodextrin; Glucog: glycogen.

Table 3.4 Diversity indices of microbial communities in soil of paddies ORG and CONV, over the rice cycle.

	Bacterial Diversity				Functional Diversity			
	Shannon index (*H*)		Evenness (*E*)		Shannon index (*H*)		Evenness (*E*)	
	ORG	CONV	ORG	CONV	ORG	CONV	ORG	CONV
April	1.26 ± 0.02	1.24 ± 0.03	0.93 ± 0.02	0.91 ± 0.02	1.44	1.44	1.00	0.99
May	1.28 ± 0.04	1.26 ± 0.06	0.94 ± 0.01	0.92 ± 0.02	1.43	1.43	1.00	1.00
June	1.27 ± 0.03	1.25 ± 0.05	0.93 ± 0.02	0.93 ± 0.03	1.45	1.44	1.00	0.99
October	1.29 ± 0.03	1.27 ± 0.06	0.95 ± 0.01	0.91 ± 0.03	1.44	1.44	1.00	1.00

No significant differences were observed over the sampling period ($P \geq 0.05$).

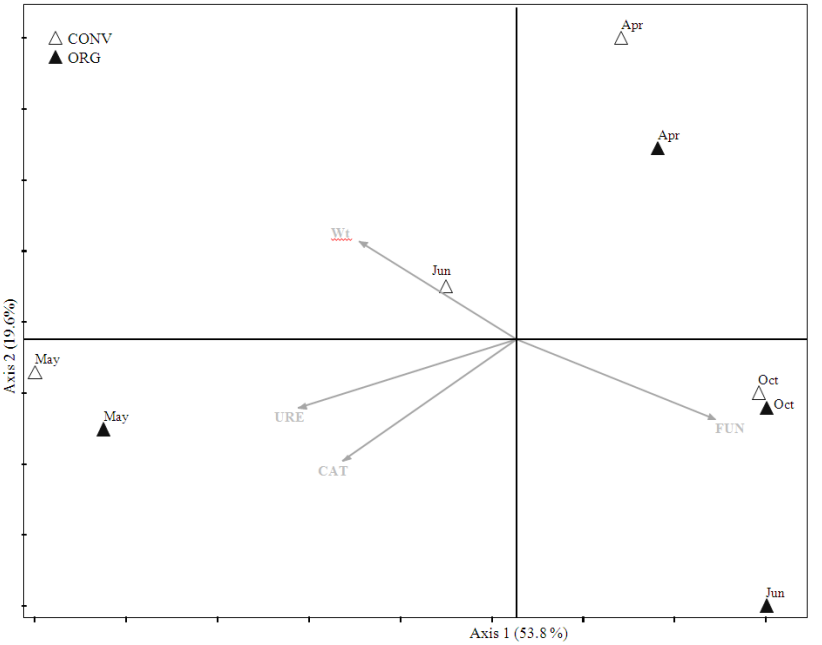

Fig. 3.3 Canonical correspondence analysis biplot of CLPP of soil from paddies ORG and CONV and physical, (bio)chemical and microbiological parameters (represented by arrows) throughout the rice cycle.

CAT, catalase activity (mmol H_2O_2 g^{-1} h^{-1}), URE, urease activity (µmol NH_3 g^{-1} h^{-1}); Wt, water content (%); FUN fungi counts (log CFU g^{-1}drysoil).

3.4.5 DGGE analysis of soil bacterial community

The analysis of DGGE profiles based on the diversity of 16S rRNA bacterial gene sequence revealed complex band patterns for both paddies and for every analysed period. Considering the totality of the DGGE profiles, a total of 38 well resolved bands were detected and assigned, with, in average, 23 bands in each DGGE profile. In general, the number of bands detected simultaneously at different sampling dates was higher in paddy CONV (13 bands) than in paddy ORG (6 bands), and among these, only two were common to both paddies and persistent over time. Most of the bands were detected in both paddies, although at different sampling periods, or with different intensities. Cluster analyses of DGGE profiles of both soils showed the separation between the paddy ORG and paddy CONV bacterial communities (Fig. 3.4).

The Shannon index of diversity (*H*) and the evenness index (*E*) values to evaluate the bacterial diversity are shown in Table 3.4. Shannon' index presented an average value of 1.28 and 1.26, for paddies ORG and CONV, respectively. The *E* values ranged between 0.93 and 0.95 for paddy ORG and between 0.91 and 0.93 for paddy CONV. The analysis of variance of these indices did not reveal significant differences within each paddy for the different analysed periods, or between them in each sampling time.

In the CCA ordination analysis of DGGE band patterns, the total variance of the data explained by axes 1 and 2 was 20.7 % and 12.2 %, respectively. Both axes presented high species-environment correlation values (0.966 and 0.899, for axis 1 and 2, respectively) (P = 0.001). The CCA biplot allowed the distinction of four groups (I-IV) of samples (Fig. 3.5). Group I enclosed all the paddy ORG patterns from May and June. Group II was constituted by all paddy CONV patterns, except two samples from June and two from October. Group III included all the paddy ORG patterns from October. Finally, Group IV included paddy ORG patterns from April. The activity of catalase and invertase, as well as the heterotrophic counts, which presented an intraset correlation of, respectively, -0.698, 0.478 and -0.456

with axis 1 contributed to separate group I from all the others. On the other hand, pH (H_2O), urease activity and molinate concentration, which presented an intraset correlation of, respectively, -0.682, 0.595 and 0.496 with axis 2 contributed to delineate groups II, III and IV.

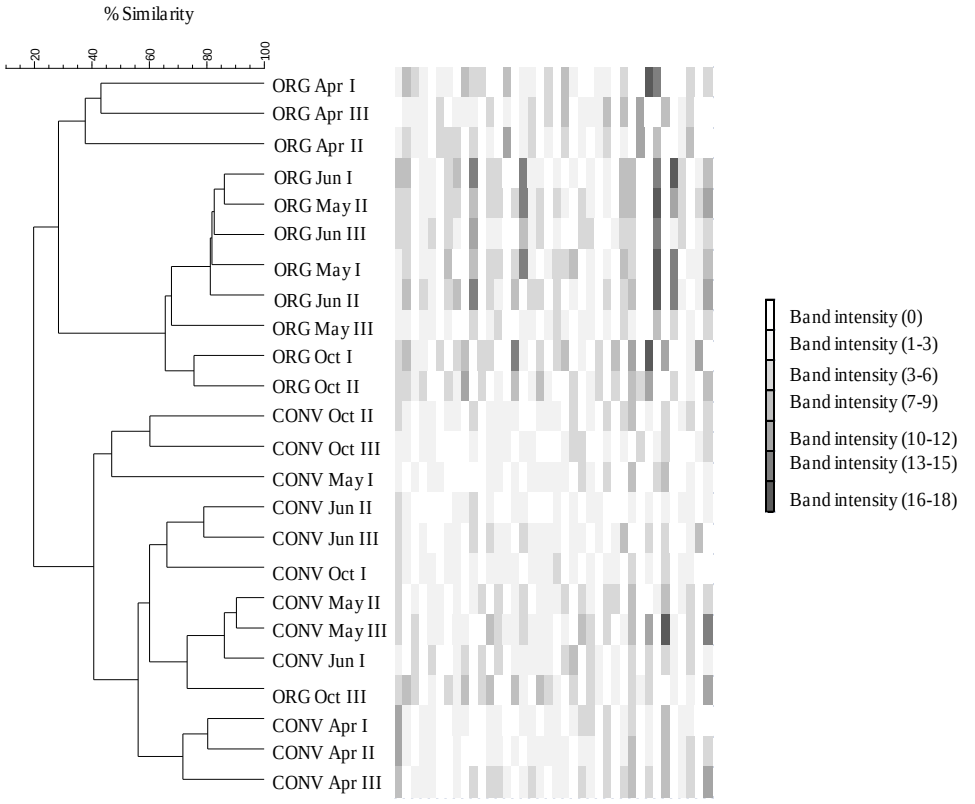

Fig. 3.4 Similarity dendrogram based on Pearson´s correlation coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) of DGGE profiles of PCR-amplified 16S rDNA fragments of bacterial communities from paddy soils ORG and CONV.

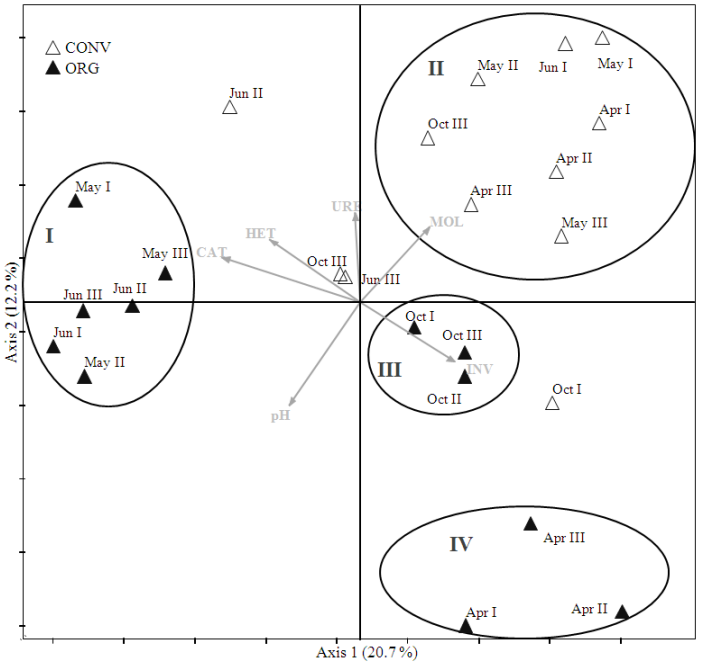

Fig. 3.5 Canonical correspondence analysis biplot of DGGE patterns of soil from paddies ORG and CONV and physical, microbiological and (bio)chemical parameters (represented by arrows) throughout the rice cycle.

CAT, catalase activity (mmol H_2O_2 g^{-1} h^{-1}); URE, urease activity (µmol NH_3 g^{-1} h^{-1}); INV, invertase activity (µmol glucose $g^{-1}h^{-1}$), HET, heterotrophs counts (log CFU g^{-1}dry soil); pH, pH H_2O; MOL, molinate concentration (mg kg^{-1}soil).

3.5 Discussion

Several physicochemical, biochemical and microbiological parameters were determined concurrently for paddies ORG and CONV over the rice cycle. With these analyses we aimed at assessing possible temporal variations for each of the studied agricultural procedures and the influence of the agricultural management on the soil properties. The agricultural management of paddy field ORG included a period (April-late May) of weeds growth. This is part of the management process in which weeds overgrowth during rice cultivation is prevented by its previous

growth and subsequent mechanical removal. Thus, from April to June both weeds and rice plants grew in this field. Given the management of paddy field CONV, for this soil, the samples from April to June corresponded to different stages of the rice growth, and included the phytosanitary treatments (molinate in May and propanil and bentazone in June) and synthetic fertilization (in June). In both paddies, the samples of October corresponded to upland conditions, after harvesting. Among the biochemical and physicochemical parameters and microbiological counts determined between April and October, a similar trend of variation was observed for both paddies. In general, independently of the management practice, enzymatic activity reached the maximum in May and the cultivable organisms in June. In October the values of most of the measured parameters were, in general, lower than in May and/or June, but higher than in April.

The CLPP and DGGE profile-based richness of soil of paddies ORG and CONV were similar over the rice cycle, with all the sole C substrates being used for growth and the same average number of DGGE bands being present at all the sampling periods for both paddies. Moreover, the Shannon and the evenness diversity indices based on the CLPP and DGGE profiles did not vary in each paddy over time or differed between paddies at each sampling time. These results suggest heterogeneity and co-abundance of different organisms in rice soil with high functional diversity, irrespectively of farming type. In fact, the high functional diversity of rice field soil was previously reported (Zhou et al. 2008), and it is known that this highly heterogeneous habitat favours the growth and survival of versatile bacterial members. Hence, these organisms contribute to a diverse community, which contrasts with the restricted bacterial communities observed in percolating water and rice roots (Asakawa and Kimura 2008; Kikuchi et al. 2007).

Given the fact that similar diversity indices were observed in both paddies, together with an identical over time trend of variation of the analysed parameters, it could be argued that the type of agricultural management practice did not have a major influence on the functional and microbial communities of the rice soil. In fact,

among the microbial counts and the biochemical and physicochemical parameters determined, only a few were observed to differ significantly between both management systems. Some of the differences found, as the water content in May and June, were probably due to field management, rather to intrinsic soil properties. The scarcity of other studies comparing conventional (pesticides application and synthetic fertilization) and organic rice farming (no pest control, mineral and organic amendments) limits a deeper discussion of our data. However, the parameters that differed between paddies ORG and CONV (pH, the activity of some enzymes and the total cell counts) are reported in literature as being susceptible of variation between organically and conventionally farmed soils. The higher values of pH registered for paddy ORG than for paddy CONV, particularly in April, is in accordance to that described in the literature when comparing different agricultural management procedures. In fact, in previous studies, organically farmed soils present slightly higher pH values than conventionally managed soils (e.g. Mader et al., 2002; Liu et al., 2007) . Several authors reported that organic management practices may lead to increased soil microbial biomass, activity and microbial functional and taxonomic richness and diversity when compared to conventional farming (Carpenter-Boggs et al. 2000; Mäder et al. 2002; van Diepeningen et al. 2006). Nevertheless, none of the previous studies referred to above have focused on rice cultures. In our study, no differences were found between both managements in what respects functional and bacterial community richness and diversity. Additionally, assuming that the microbial biomass can be related to the microbial counts, the results obtained in the present study for rice soil did not follow the pattern described by other authors, who compared different agricultural procedures for other crops (Carpenter-Boggs et al. 2000; Mäder et al. 2002; van Diepeningen et al. 2006). In contrast, rice field soil studies of Sheng et al. (2005), although not comparing organic with conventional farming, are in agreement with ours. Given the uniqueness of the rice culture, which includes a long period of flooded conditions, it would not be surprising that farming management might trigger different responses in soil communities, than

those observed in other crops. In a comparative study of the effects of synthetic fertilization and/or pesticides use, Sheng et al. (2005) reported that the microbial biomass and the number of heterotrophic bacteria in rice field soil subjected to incorporation of synthetic fertilizers alone was not significantly different from rice field soil under conventional farming (synthetic fertilization and pesticides), although enhanced dehydrogenase activity was observed in the first soil. Additionally, Sheng et al. (2005) observed that the highest number of heterotrophs in both soils was at tillering stage, which is in agreement with our study, as in June rice plants were at the same growth stage. Interestingly, the highest MPN of microorganisms growing in the sole C substrates tested was coincident with the highest counts of fast growing heterotrophs and actinomycetes. This observation may be related with enhanced availability of labile C and N in both soils in June. In this period, rice plants were growing towards maturation in both paddies, and the highest density of cultivable bacteria and microorganisms growing in the tested carbohydrates, amino acids and organic acids, compounds present in root exudates (Kong et al. 2008), would support this observation. In fact, photosynthesized compounds released from rice roots to the soil can provide nutrients to increase the microbial populations (Bai et al. 2000). On the other hand, the addition of synthetic N fertilizers in paddy CONV may have also contributed to enhance the growth of the cultivable soil populations. Thus, the influence of root exudates may have been noticeable in paddy ORG because it did not receive fertilization or any other treatment after seeding.

Multivariate analysis of soil fingerprints is a useful tool to detect shifts in the microbial composition of organically and conventionally farmed soils, as well as temporal variations in each type of farming system. For instance Hartmann et al. (2006) reported significant differences in the microbial communities, characterized on basis of T-RFLP profiling, of organically and conventionally managed soils. However, the observed shifts were mainly due to the incorporation of farmyard manure (FYM), as the microbial communities of organically and conventionally managed soils with FYM amendment were similar. In a further study, Hartmann

and Widmer (2006) reported highly similar microbial diversity indices values among unfertilised, organic and conventional FYM fertilised soils, whereas through the analysis of the composition of the communities by T-RFLP profiling and by sequence of 16S rRNA genomic libraries strong differences in composition were found. These findings led to the conclusion that changes in the microbial community structure may not lead to shifts in the diversity indices values, since some taxonomic groups may be compensated by modifications of others (Hartmann and Widmer 2006). In another study, Bossio et al. (1998) detected highly significant differences in PLFA profiles between organically and conventionally farmed tomato soil plots and among sampling dates, while the Shannon's diversity index values based on PLFA relative abundance did not vary between farming system type neither over time. This observation, as in our study, may indicate the limitation of the Shannon's diversity index to assess microbial diversity on basis of fingerprinting data. Nevertheless, Fromin et al. (2002) in their comprehensive review on community analysis by DGGE patterning, refers this index as a valuable tool. These observations reinforce the relevance of multivariate analyses to infer about temporal variations or farming systems. In fact, such analyses allowed an integrated interpretation of the data and revealed that the soil functional community structure presented similar temporal variations irrespective of the type of management, while the farming type influenced the bacterial community structure.

The CLPP patterns of paddies ORG and CONV varied over time in a similar manner, except for June. Parameters as the urease activity, which peaked in May, and the fungi counts, which presented high values in October, for both paddies may explain the separation of the functional community structure in these months. The catalase activity, which showed the lowest values in April, influenced the separation of the CLLP patterns of both paddies in this month. Dissimilar functional community structure in paddies ORG and CONV was mainly explained by the significant differences in the soil water content found in these paddies in June. Although also varying over time, in paddy ORG the DGGE patterns-based

groups were slightly different from the CLPP-based groups. In this case, the bacterial community of May and June grouped together. The high catalase activity values and counts of heterotrophs found in May and June, and the high invertase activity values registered in April and October contributed to explain the variation observed between the communities of May/June and those of April and October. The lowest urease activity values and the highest values of pH influenced the separation of the DGGE profiles of paddy ORG in April. Similar results were obtained by Kikuchi et al. (2007) and by (Watanabe et al. 2006; 2007) when analysing, respectively, the bacterial and methanogenic populations of rice soils with synthetic fertilization through PCR-DGGE rDNA or RT-PCR rRNA profiling over time. As we observed, those authors reported that although the number of DGGE bands did not fluctuate throughout the year, multivariate analysis showed that differences in bands intensity were sufficient to demonstrate that the period of rice cultivation influenced the bacterial and methanogenic communities (Kikuchi et al. 2007; Watanabe et al. 2006). Although the agricultural management of rice crop is not described by Min-Cheol et al. (2008), these authors reported also temporal variations in the microbial community based on PLFA profiles and of 16S rDNA clone libraries of rice field soil. Interestingly, the temporal variation in cultivable bacteria, actinomycetes and fungi described by Min- Cheol et al. (2008) between rice planting and one month after harvest is similar to that observed in the present study.

The DGGE profiles of paddy CONV, which presented a higher number of common bands at every analysed period than paddy ORG, formed a distinct group, which was correlated, among other factors, with the presence of molinate in the soil. When compared to paddy ORG, the bacterial community of the paddy CONV did not show temporal variation, suggesting that the bacterial community structure of both paddies was distinct at each sampling time. In fact, the cluster analyses of DGGE profiles of both paddies run in the same gel, for each of the analysed periods, showed that the DGGE patterns of paddies ORG and CONV always formed separated clusters (data not shown). The influence of the agricultural

management procedures on the structure of the soil bacterial communities was, thus, demonstrated in the current study. Nevertheless, differences in land use may also have contributed to differentiate the bacterial community structure of both paddies, as paddy ORG was under production for only two years while paddy CONV was for six years. This study also evidenced the higher plasticity of the organically farmed rice soil bacterial community, given its higher responsiveness to temporal shifts than that observed in the conventionally farmed paddy.

In summary, it was observed that the microbial populations of rice field soil subjected to organic and conventional farming presented similar functional and bacterial richness and diversity, with temporal variations on the microbial density (cell counts), CLPP and enzymatic activity. Given these parameters may reflect the metabolic capabilities of the fast growing fraction of the bacterial community, our results suggest that this bacterial fraction seems to be less affected by the agronomic management than the total bacterial community, that was shown to differ between organically and conventionally farmed soil. On the other hand, functional redundancy of soil microorganisms may also explain the observed results.

Chapter 4

Molinate biodegradation in soils: natural attenuation *versus* bioaugmentation

Results included in:

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4.1 Abstract

The aims of the present study were to assess the potential of natural attenuation or bioaugmentation to reduce soil molinate contamination in paddy field soils and the impact of these bioremediation strategies on the composition of soil indigenous microbiota. A molinate mineralizing culture (mixed culture DC) was used as inoculum in the bioaugmentation assays. Significantly higher removal of molinate was observed in bioaugmentation than in natural attenuation microcosms (63 and 39 %, respectively) after 42 days of incubation at 22 °C. In the bioaugmentation assays, the impact of *Gulosibacter molinativorax* ON4^T on molinate depletion was observed since the gene encoding the enzyme responsible for the initial molinate breakdown (harboured by that actinobacterium) was only detected in inoculated microcosms. Nevertheless, the exogenous mixed culture DC did not overgrow as the heterotrophic counts of the bioaugmentation microcosms were not significantly different from those of natural attenuation and controls. Moreover, the actinobacterial clone libraries generated from the bioaugmentation microcosms did not include any 16S rRNA gene sequences with significant similarity to that of *G. molinativorax* ON4^T. The multivariate analysis of the 16S rRNA DGGE patterns of the soil microcosm suggested that the activity of mixed culture DC did not affect the soil bacterial community structure since the DGGE patterns of the bioaugmentation microcosms clustered with those of natural attenuation and controls. Although both bioremediation approaches removed molinate without indigenous microbiota perturbation, the results suggested that bioaugmentation with mixed culture DC was more effective to treat soils contaminated with molinate.

Keywords: Natural attenuation, Bioaugmentation, Molinate, Bacterial community, Paddy field soil

4.2 Introduction

Thiocarbamate molinate is an herbicide widely used for the control of barnyard grass in paddy fields. Contamination of soil, leachate and receiving waters by molinate has been observed in various countries at levels up to 100 μg l^{-1} (Carrasco et al. 1987; Julli and Krassoi 1995; Mabury et al. 1996). Environmental contamination with molinate is a concern because the herbicide and some of its degradation products have adverse effects on humans and animals (Cochran et al. 1997; Ellis et al. 1998; Golovleva et al. 1981; Jewell et al. 1998; Jewell and Miller 1999). Therefore, strategies to remediate this contamination are necessary.

The cost-effectiveness of bioremediation when compared with other physicochemical remediation processes makes it attractive. Bioremediation relies on the metabolic diversity and cooperation of microorganisms, leading to the breakdown of different organic pollutants and degradation products thereof (Wackett and Hershberger 2001). Given its dependence on microbiological activity, the improvement and reliability of the bioremediation processes require the characterization of the microorganisms and microbe–microbe and environmental interactions (Bombach et al. 2010; Röling and van Verseveld 2002). Factors such as the pollutant properties, temperature, availability of nutrients, and electron acceptors will influence the rate and extent of biodegradation and, thus, the success of the bioremediation processes (Hussain et al. 2009; Reid et al. 2000; Röling and van Verseveld 2002; Vidali 2001; Vogel 1996). In spite of the high potential of autochthonous microorganisms to eliminate pollutants (natural attenuation), the extent of biodegradation may only be residual. For this reason, bioaugmentation is sometimes preferred. In bioaugmentation processes, soils are inoculated with microorganism(s) able to degrade the pollutant. Successful implementation of bioaugmentation depends on the efficiency of the added culture to degrade the pollutant under natural conditions and to adapt to the indigenous microbiota (Mrozik and Piotrowska-Seget 2010; Thompson et al. 2005; Vogel 1996). In fact, the competition between exogenous and indigenous microbiota may

be responsible for unsuccessful bioaugmentation processes (Bouchez et al. 2000; Olaniran et al. 2006).

Although molinate is considered as one of the most recalcitrant thiocarbamates (Nagy et al. 1995), biodegradation in soils by aerobic microorganisms (bacteria and fungi) through co-metabolism is reported (Golovleva et al. 1981; Imai and Kuwatsuka 1982; Imai and Kuwatsuka 1986a; Skryabin et al. 1978; Thomas and Holt 1980). Up to now, only one microbial culture (mixed culture DC) has been described as being able to mineralize molinate as the sole source of carbon, nitrogen and energy under a wide variety of operating conditions (Barreiros et al. 2003; Correia et al. 2006). The molinate degradation pathway used by culture DC is different (Barreiros et al. 2008) from others previously reported (Golovleva et al. 1981; Imai and Kuwatsuka 1982; Thomas and Holt 1980). The novel pathway is explained in part by the activity of molinate hydrolase (MolA) which is encoded by *Gulosibacter molinativorax* ON4^{T}, member of culture DC, and that is responsible for the initial breakdown of the herbicide (Barreiros et al. 2008; Duarte et al. 2011). MolA leads to the breakdown of molinate into ethanethiol and azepane-1-carboxilate, which are further mineralized by a metabolic cooperation among the five members of mixed culture DC, *G. molinativorax* ON4^{T}, *Pseudomonas* (two strains), *Stenotrophomonas* and *Achromobacter* (Barreiros et al. 2008).

The aim of the present study was to assess the potential for molinate biodegradation under either natural attenuation or bioaugmentation processes. Additionally, it was also intended to characterize the effects of molinate and inoculation with an exogenous inoculum (mixed culture DC) on the indigenous microbiota. The effectiveness of natural attenuation and bioaugmentation was evaluated using ex situ microcosms with soil samples from an organically farmed rice paddy field spiked with molinate.

4.3 Materials and methods

4.3.1 Microcosm assays

Microcosm assays were prepared with soil samples collected from an organically farmed Portuguese rice paddy field from the experimental farm "Bico da Barca" (40 º11′N; 08 º41′W) in April of 2007. A composite sample was prepared by mixing identical slots of three different sub-samples, each consisting of three soil cores pooled together from the upper 0–15 cm of the soil and stored at 4 °C until use. The soil pH was 6.4 and its total C and N content was 1.9 and 0.2 %, respectively. Other soil characteristics and description of the agricultural management of the rice paddy are described elsewhere (Lopes et al. 2011). Prior to use, the composite samples were sieved (<2 mm) and air-dried. Soil sterilization was done by autoclaving at 121 °C for 1 h for three consecutive days.

Natural attenuation was assessed in microcosms with soil spiked with molinate (M) (natural attenuation = M). Bioaugmentation was assessed under similar conditions, with soil inoculated (i) with mixed culture DC (bioaugmentation = Mi) (Barreiros et al. 2008; Barreiros et al. 2003). Abiotic losses were assessed in microcosms with non-inoculated sterile soil (s) spiked with molinate (CsM). The ability of mixed culture DC to grow and degrade molinate in soil was assessed in microcosms with inoculated sterile soil spiked with molinate (CsMi). Non-sterile and non-inoculated microcosms were used to assess the impact of the experimental setup (microcosms) on the indigenous bacterial community (*C*). The effect of mixed culture DC on the indigenous bacterial community was assessed in inoculated non-sterile microcosms (Ci).

4.3.2 Setup of microcosms

Microcosms were prepared by placing 2 g of homogenized moist soil into 25-ml sterile glass vials with Teflon-lined caps and incubated at 22 °C at static conditions for 42 days.

The homogenized soil was prepared as follows: soil amendment was performed by mixing non-sterile (M, Mi) or sterile soil (CsM, CsMi) with a sterile molinate aqueous solution for 1.5 h at 120 rpm, reaching a final moisture and molinate content of 45 % and 0.1 mg molinate g^{-1}dry soil,respectively. A sterile water solution was used for microcosms that did not contain molinate (*C* and Ci) in order to obtain a final moisture content of 45 %.

To inoculate the microcosms, mixed culture DC was grown in mineral medium B containing 4 mM of molinate as the only source of carbon, nitrogen and energy (Barreiros et al. 2003). At the end of the exponential growth phase, the number of total cells was estimated using the 4,6-diamidino-2-phenylindole staining method as previously described (Manuel et al. 2007). This culture was suspended in a molinate solution (for Mi, CsMi) or sterile water (for Ci) in order to obtain an initial cell density of approximately 2×10^5 $cells_{mixed\ culture}$ g^{-1}dry soil, which corresponded to 10 % of the total number of heterotrophs per gram of dry soil.

4.3.3 Sampling from microcosms

For molinate-spiked microcosms, six independent microcosms were sacrificed for each treatment at days 0, 28 and 42 for M, Mi and CsMi (total number of microcosm per treatment was 18) and at days 0 and 42 for CsM (the total number of microcosms per treatment was 12). Three of the sacrificed microcosms were used to determine the molinate concentration and the other three were used to enumerate cultivable heterotrophs and for further molecular characterization. For comparison, the enumeration of cultivable heterotrophs and molecular characterization was also made in microcosms not spiked with molinate (*C* and Ci) sampled at days 0 and 42 (the total number of microcosms per treatment was 6). Quantification of molinate and cultivable heterotrophs were performed immediately after sampling. The samples used for soil molecular characterization were stored at -20 °C until processing.

4.3.4 Soil analysis

The soil molinate content was determined after extracting twice with 4 ml of hexane. Prior to extraction, samples (2 g moist soil) were spiked with an internal standard to achieve a final concentration of 0.1 mg cycloate g^{-1}dry soil. The organic extracts were pooled, dried under vacuum, resuspended in 0.85 ml methanol and analysed by high performance liquid chromatography as described by Barreiros et al. (2003).

Fast-growing cultivable heterotrophs were enumerated using the plate count method. Aliquots of 0.5 g of microcosm soil were suspended in 4.5 ml of sterile saline solution (0.85 % NaCl) and serially diluted, spread (100 µl) onto Luria–Bertani agar plates and incubated at 30 °C for 48 h.

The soil molinate content and heterotrophic plate counts were expressed as mg of molinate or colony forming units (CFU) g^{-1}dry soil $^{-1}$ (oven-dried soil basis), respectively, corresponding to the average value of three independent soil microcosm analyses.

4.3.5 DNA extraction from soil microcosms

Total genomic DNA was extracted from 0.3 g of microcosm soil using the Power Soil™ DNA Isolation Kit (MO BIO) using a modified version of the protocol described by Lopes et al. (2011). Briefly, samples were sonicated (bath sonication) for 5 min, agitated at 1,300 rpm for 20 min and incubated at 65 °C for 15 min. Genomic DNA quality and quantity was assessed as previously described (Lopes et al. 2011).

4.3.6 Bacterial community analysis

The structure of microcosm soil bacterial communities (M, Mi, *C* and Ci) at days 0 and 42 were analysed by denaturing gradient gel electrophoresis (DGGE) of the

amplified 16S rRNA gene fragment. PCR amplification of bacterial 16S rDNA fragments (~500 bp) was performed using the universal bacterial primers F984-GC and R1378 (Heuer et al. 1997; Nubel et al. 1996) as previously described (Lopes et al. 2011). The PCR products were quantified (Qubit® Fluorometer) and ~75 ng of the amplified 16S rDNA fragments was separated in a double-gradient polyacrylamide gel containing 6–9 % acrylamide with a denaturing gradient ranging from 30 to 58 % (where 100 % denaturant contained 7 M urea and 40 % formamide) as previously described (Lopes et al. 2011).

4.3.7 Detection of molinate hydrolase gene (*mol*A) in soil microcosms

The presence of the MolA-encoding gene was assessed in soil microcosm samples by PCR using the primers F94 (5'-CAGGATCACGAAGGTTGGTT-3') and R1122 (5'-ATCCACACGAAGTGGTCCTC-3') (*G. molinativorax* ON4^{T} numbering, accession number FN985594) (Duarte et al. 2011). Detection of *mol*A gene was performed in M, Mi and Ci microcosms at days 0 and 42 and at day 42 for CsMi microcosms. The reaction mixture (25 µl) was composed of 1× buffer with 25 mM KCl and 10 mM $(NH_4)_2SO_4$ (Fermentas), 0.3 mM dNTPs, 4 mM $MgCl_2$, 5 % DMSO, 0.4 mg ml^{-1} of bovine serum albumin, 0.4 µM of each primer, 0.75 U taq DNA polymerase (Fermentas) and 2 µL of target DNA. The thermal cycling conditions consisted of a first denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C (0.5 min), 55 °C (0.5 min) and 72 °C (1.75 min), with a final 20-min extension at 72 °C. The PCR products were visualized in a 1.5 % agarose gel stained with ethidium bromide. Positive reactions (*mol*A+) were indicated by the presence of an amplicon of approximately 1.1 kb. The authenticity of the *mol*A gene was confirmed by sequencing analysis of the PCR product. The PCR product was purified (Purification Kit, GFX™ PCR DNA and Gel Band Purification Kit, Amersham Biosciences, NJ, USA), ligated into pTZ57RT/T vector (InsTAclone™ PCR cloning kit, MBI Fermentas, Heidelberg, Germany) and used to transform *Escherichia coli* JM109 (NZYTech, Lisbon, Portugal) according to the

manufacturer's instructions. The DNA of insert-positive clones was sequenced with the universal vector primers M13pUC-F (5'-GTTTTCCCAGTCACGAC-3') and M13pUC-R (5'- CAGGAAACAGCTATGAC-3'). Nucleotide sequences were determined using a model ABI 3700 DNA Analyser (Applied Biosystems, CA, USA). After checking their quality using the BioEdit software (Hall 1999), the nucleotide sequences (ca. 1,100 bp) were compared to others available in public databases using the BLAST search tool (http://www.ncbi.nlm.nih.gov).

4.3.8 Detection of *G. molinativorax* ON4^T in soil microcosms

To assess the effect of *G. molinativorax* ON4^T on the soil actinobacterial communities, 16S rDNA clone libraries from M, Mi, CsMi, C and Ci microcosms at days 0 and 42 were prepared. Given that 16S rDNA actinobacterial specific primers F243HGC and R1378 described by Heuer et al. (1997) failed on the amplification of *G. molinativorax* ON4^T, a modified forward primer (F243GM, 5'-GGAT*C*AGCC C*A*CGGCCTA-3') was designed. In primer F243GM, two nucleotides (fifth and 11th, italicized in the preceding sentence) were substituted in comparison to primer F243HGC (Heuer et al. 1997). These nucleotides are characteristic of *G. molinativorax* ON4^T and *Pseudoclavibacter helvolus* DSM 20419^T. PCR reactions were performed in a 50-µl reaction mixture with a composition identical to that reported earlier, differing on the use of 3 mM $MgCl_2$, 0.3 µM of each primer, 1.5 U taq DNA polymerase and 3 µL of target DNA. The PCR program was 5 min at 94 °C, followed by 35 cycles of 1min at 94 °C, 1min at 52 °C, 2 min at 72 °C and a last step of 20 min at 72 °C. The amplicons (~1.1 kb) obtained from the triplicate microcosms at days 0 or 42 were pooled before gel purification (GFX™ PCR DNA and Gel Band Purification Kit, Amersham Biosciences, NJ, USA). Cloning of gel-purified amplicons was performed and the DNA of insert-positive clones was subsequently sequenced with primer M13pUC-F as described above.

The 16S rRNA gene sequences' quality was checked using the BioEdit software (Hall 1999) and were aligned using Clustal W from MEGA 5.0 software (Tamura et al. 2011). The sequences retrieved from the clone libraries were processed separately for each microcosm assay. Sequences sharing > 98 % similarity were considered as corresponding to the same operational taxonomic unit (OTU). The OTU identity was assigned based on the EzTaxon library (http://www.eztaxon.org/) (Chun et al. 2007).

4.3.9 Statistical analysis

Statistical analysis (two-way analysis of variance-ANOVA and two-sample statistical test-t-test) of molinate degradation and cultivable heterotrophs data was performed using the Microsoft Excel software package.

Analyses of bacterial community profiles in the soil microcosms were performed with the Bionumerics software (version 6.1, Applied Maths, Sint-Martens-Latem, Belgium) after normalization of scanned DGGE gels with a standard reference. The DGGE profiles of all analysed samples were compared after band-matching in the area 15.6–85.3 % of the gel. The obtained band-matching tables were the basis for community cluster and ordination analysis and for the determination of diversity indices [$H = -\Sigma(n_i/N)\log(n_i/N)$] (Shannon and Weaver 1963) and [$E = H/\log S$] (Pielou 1966). Canonical correspondence analysis (ter Braak 1986) was carried out to evaluate the influence of soil molinate amendment and of abundance of the heterotrophic population on the variance of the bacterial community composition (DGGE patterns). CCA was performed with CANOCO (version. 4.5, Microcomputer Power, Ithaca, NY, USA). Monte Carlo permutation test (n=999) was used to evaluate the significance of the relationship between community and environmental data.

4.3.10 Nucleotide sequence accession numbers

Representative actinobacterial 16S rDNA sequences obtained in this study were deposited in GenBank with accession numbers JQ669500 to JQ669509.

4.4 Results

4.4.1 Molinate degradation and total heterotrophic counts in soil microcosms

Molinate was biodegraded either by natural attenuation or bioaugmentation processes. Abiotic losses of molinate did not exceed 4 % (CsM microcosms) (Table 4.1). At day 42, a higher herbicide removal ($P<0.01$) was observed in Mi (~63 %) than in M (~39%) microcosms. The highest molinate depletion was observed in the inoculated control CsMi (~99 %).

In general, molinate depletion was accompanied by an increase in the total number of soil heterotrophs (Fig. 4.1a). At the beginning of the experiments (day 0), the total heterotrophic counts were similar in both M and Mi microcosms (~6.2 and 6.5 log CFU g^{-1}dry soil, respectively). The slight difference observed was due to the presence of the inoculum, which corresponded to ~10 % of the total number of soil heterotrophs. In the same way, at the end of the incubation period (42 days), the total heterotrophic counts were not significantly different in both M and Mi microcosms (~7.2 and 7.3 log CFU g^{-1}dry soil, respectively). Identical values were found in both non-inoculated and inoculated non-spiked controls (*C* and Ci) (Fig. 4.1a). In contrast, at day 42, significantly higher counts were observed in CsMi (9.0 log CFU g^{-1}dry soil) than in all the other microcosms. In fact, the difference in the number of heterotrophs between CsMi and the other microcosms was approximately two orders of magnitude.

Table 4.1 Molinate concentration over time and percentage of degradation in microcosm assays.

Time (days)	Molinate concentration (μg g^{-1}dry soil) (% of degradation)			
	M	Mi	CsM	CsMi
0	108 ± 15 (0)			
28	75 ± 28 (31 ± 25)	65 ± 6 (40 ± 5)	N.D.	18 ± 29 (83 ± 27)
42	66 ± 16 (39 ± 15 ^{B})	40 ± 8 (*63* ± *7* ^{C})	104 ± 4 (4 ± 4 ^{A})	2 ± 1 (99 ± 1 ^{D})

The initial concentration of molinate in soil microcosms is an average value of all the samples ± standard deviation (n=12); all the other values are means ± standard deviation (n=3). Significantly different values of molinate depletion are indicated by letters A, B, C or D for distinct treatments ($P < 0.01$) or by italics for different sampling dates (28/42 days) ($P < 0.05$). N.D. not determined

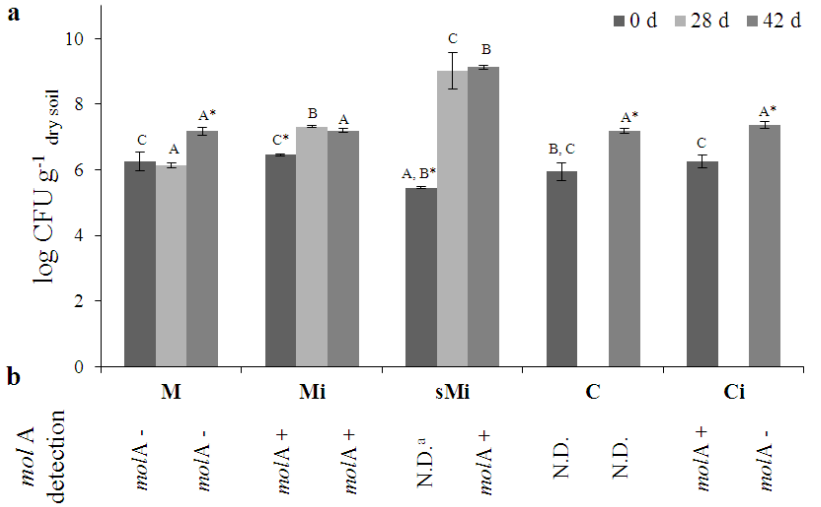

Fig. 4.1 a) Total heterotroph counts (log CFU g^{-1}dry soil) in M, Mi, CsMi, *C* and Ci microcosms over the incubation period. Results are mean values (n=3) and the error bars represent the standard deviation. **b)** Detection of *mol*A gene in DNA extracted from M, Mi, CsMi and Ci soil microcosms from days 0 and 42.

Significantly different values of total heterotrophs are indicated by A, B or C for distinct treatments ($P < 0.01$) or by an asterisk for different sampling dates (0/28/42 days) ($P < 0.05$). *mol*A + gene was amplified, *mol*A − gene was not amplified, N.D.a not determined since it was not possible to extract DNA from that sample, N.D. not determined.

4.4.2 Bacterial community structure in soil microcosms

The structure of soil bacterial communities from M, Mi, *C* and Ci microcosms and their temporal variations were analysed by 16S rDNA-DGGE. On average, DGGE patterns comprised 14 and 16 well-resolved bands at days 0 and 42, respectively. Variations in the structure of the bacterial communities based on the DGGE patterns of the four microcosms were characterized both by cluster analysis (data not shown) and canonical correspondence analysis (Fig. 4.2). Axis 1 explained 27.1 % of the observed variance and presented a high species–environment correlation value (0.94) ($P<0.02$). As indicated by the CCA biplot, the structure of the bacterial communities varied over the incubation period (days 0 and 42) in all of the analysed microcosms. At the end of the incubation period (day 42), the bacterial communities of non-inoculated and inoculated non-spiked controls (*C* and Ci) were closer to M and Mi microcosms than at the beginning of the experiment (day 0) (Fig. 4.2). The number of total heterotrophs was associated with the temporal variations in the bacterial communities, showing an intraset correlation of -0.98 with axis 1 and explaining 76.7 % of the variance of the species–environment relation. Axis 2 explained 8.2 % of the variance in the bacterial communities. At the start of the experiment (day 0), the bacterial communities of the non-spiked controls (*C* and Ci) differed from those of M and Mi microcosms as indicated by their separation along axis 2. Indeed axis 2, which explained 23.3 % of the variance of species–environment relation, was related to soil molinate content (intraset correlation of -0.84).

When the same communities (M, Mi, *C* and Ci) were compared based on the diversity (*H*) and the evenness (*E*) indices, no significant differences were observed, either at the beginning or at the end of the experiment (0 or 42 days; Table 4.2).

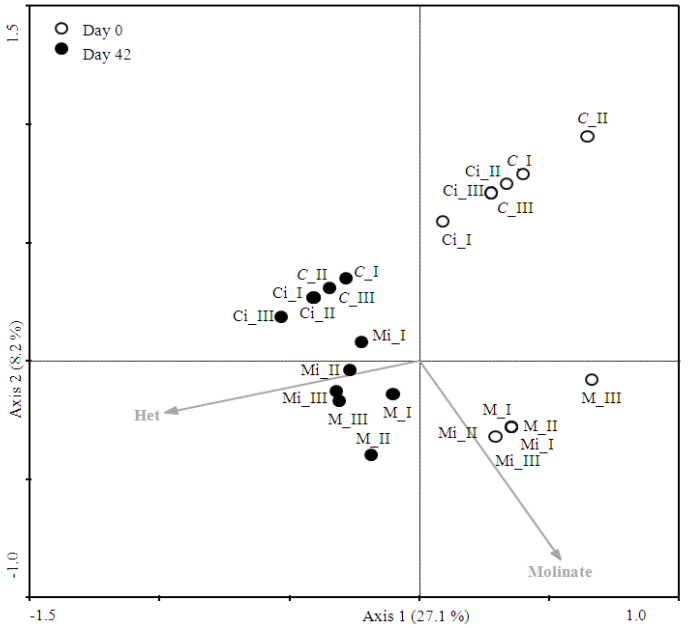

Fig. 4.2 CCA biplot of DGGE patterns of soil microcosms M, Mi, *C* and Ci over the incubation period (0 and 42 days). The total heterotrophic count and molinate concentration, which are the explanatory variables, are represented by arrows indicating the direction of increase for each variable.

Table 4.2 Diversity indices of bacterial communities over the incubation period.

Time (days)	Shannon index (*H*)			
	M	Mi	*C*	Ci
0	1.03 ± 0.02	0.96 ± 0.01	0.99 ± 0.02	0.92 ± 0.07
42	1.08 ± 0.04	1.09 ± 0.03	1.05 ± 0.04	1.08 ± 0.05

Table 4.2 Continued

Time (days)	Evenness index (*E*)			
	M	Mi	*C*	Ci
0	0.89 ± 0.01	0.83 ± 0.02	0.89 ± 0.02	0.83 ± 0.07
42	0.89 ± 0.03	0.89 ± 0.04	0.90 ± 0.03	0.92 ± 0.01

Values are means ± standard deviation (n=3)

4.4.3 Detection of *mol*A gene in microcosm soil

Molinate depletion in M microcosms suggested the presence of indigenous microbiota capable of herbicide transformation. This result leads to the screening of the gene *mol*A in the microcosm assays (M, Mi, Ci and CsMi). After numerous attempts, this gene was only detected in microcosms inoculated with culture DC and never in the M microcosms. At day 0, the gene was detected in Mi and Ci microcosms, and at day 42 *mol*A was detected from the inoculated and spiked microcosms (Mi and CsMi) (Fig. 4.1b). The nucleotide sequence of the *mol*A amplicon confirmed a high similarity with the same gene of strain *G. molinativorax* ON4^T (Duarte et al. 2011) with identity values of 99.5–99.9 % with *mol*A.

4.4.4 Detection of *G. molinativorax* ON4^T in soil microcosms

The effect of *G. molinativorax* ON4^T inoculation on the soil actinobacterial communities was assessed based on the analysis of an actinobacterial 16S rRNA gene library with the modified primer F243GM. Although this primer has two mismatches with the 16S rRNA gene sequence of *G. molinativorax* ON4^T, it could amplify the DNA fragment of this organism and of other *Actinobacteria*. In fact, an *in silico* analysis (TestProbe analysis, www.arb-silva.de) (Pruesse et al. 2007), allowing the same number of mismatches (two), indicated that primer F243GM could amplify a higher number of sequences affiliated to members of families *Streptomycetaceae*, *Mycobacteriaceae* and *Pseudonocardiaceae* (79.2, 3.3 and 3.2 %, respectively) than Microbacteriaceae (0.5 %) to which *G. molinativorax* ON4^T belongs. In addition to members of *Gulosibacter*, the 16S rRNA gene sequence fragments of members of closely related genera as *Pseudoclavibacter* and *Zimmermannella* could also be amplified.

Although *in silico* analysis revealed that primer F243GM was not totally specific for *Actinobacteria*, as expected the majority of the microcosm-cloned sequences (103/124) could be assigned to this phylum. Twenty-one sequences corresponded to unknown OTU showing similarity values below 90 % with known phyla.

Although the closest neighbors were members of the phyla *Firmicutes*, *Proteobacteria* and *Verrucomicrobia*, similarity values ranged between 85 and 90 %, which is too low to support a reliable identification. In M, Mi, *C* and Ci microcosms, the sequences assigned to *Actinobacteria* represented seven OTU, which were affiliated to the genera *Streptomyces* (five OTU), *Mycobacterium* and *Pseudonocardia*. The overrepresentation of a single OTU was evident in these microcosms (M, Mi, *C* and Ci), with 87 out of the 119 sequences analysed being affiliated to *Streptomyces shaanxiensis* CCNWHQ 0031^T (> 99 % nucleotide sequence identity). Comparing the beginning with the end of the assays (0 and 42 days), it is suggested that the incubation period leads to a decrease in the number of sequences affiliated to *S. shaanxiensis* CCNWHQ 0031^T. This effect was more (from 12/14 to 4/10) and less (from 13/14 to 15/17) pronounced in the inoculated control Ci and Mi microcosms, respectively. Sequences affiliated to *G. molinativorax* ON4^T were only obtained from the control CsMi (Fig. 4.3).

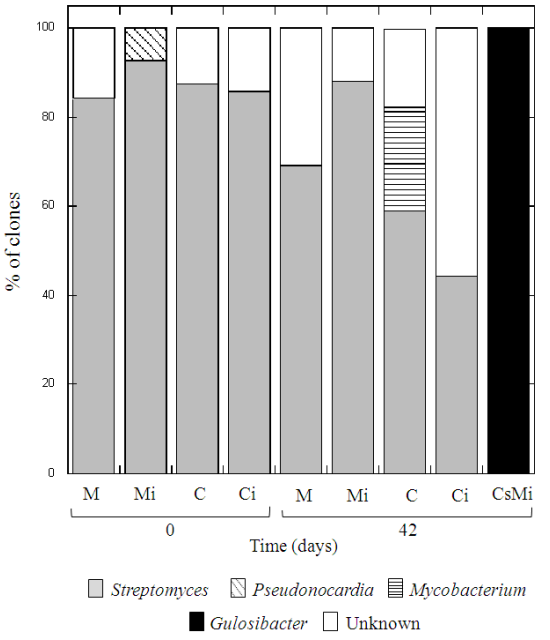

Fig. 4.3 Diversity of the actinobacterial 16S rRNA gene clones from soil microcosms.

4.5 Discussion

In the present study, the indigenous microbiota of a rice field soil was capable of molinate degradation. These results are also in agreement with other studies that used paddy soil from experimental or agriculture fields: lysimeter assays, (Park et al. 2005), microcosm assays (Imai and Kuwatsuka 1982), soil perfusion assays (Imai and Kuwatsuka 1986a) and enrichment cultures (Molinari et al. 1992). The fact that an organically farmed soil was used presumes that the microbiota has not been in contact with molinate and suggests that soil microbial community in this study may have an intrinsic capacity to transform the herbicide without a prior adaptation.

To the best of our knowledge, there is no information regarding bioaugmentation processes to reduce molinate contamination in soils. In fact, this is the first report of soil inoculation with a mixed culture able to mineralize molinate (mixed culture DC). Inoculation with that culture improved the extent of molinate depletion in soil as lower herbicide residues were found at the end of the assay in the Mi than in the M microcosms. Nevertheless, the highest molinate depletion was observed in the inoculated control in which culture DC had no competition with the indigenous microbiota (approximately 99 % in CsMi against 63 % in the Mi assay). This suggests that the ability of mixed culture DC to degrade molinate was affected by the presence of the indigenous microorganisms. Similar results were reported by Barreiros et al. (2011) in bioaugmentation microcosms using mixed culture DC to decontaminate rice paddy floodwater after the application of Ordram, a commercial molinate formulation. The result of more extensive molinate depletion in the absence of indigenous microbiota supports previous studies which showed that competition between exogenous and indigenous microbiota may compromise the successful implementation of bioaugmentation (Bouchez et al. 2000; Olaniran et al. 2006). However, in the current study, the competition between exogenous and indigenous microbiota did not hamper molinate degradation.

The depletion of molinate in non-inoculated microcosm indicated that soil indigenous microbiota has the appropriate genetic makeup to transform molinate. Nevertheless, the results suggested that, in the soil from M microcosms, the transformation pathway(s) is different from that used by mixed culture DC. Indeed the key catabolic gene involved on molinate breakdown by *G. molinativorax* ON4^T (*mol*A) was never detected in M microcosms. In contrast, *mol*A was detected in the Mi microcosms where a higher molinate removal was observed than in the M. Altogether these results suggest that at least part of molinate depletion in Mi was due to the activity of *G. molinativorax* ON4^T. Nevertheless, in the inoculated microcosms without molinate (Ci), the *mol*A gene was only detected at day 0 and not at day 42, suggesting that, in the absence of molinate, *G. molinativorax* ON4^T is not able to proliferate in soil.

The time course increase of total heterotrophic counts was similar in the M and Mi microcosms and controls (*C* and Ci). Therefore, in the current study, the increase in the heterotrophic population could not be attributed to an overgrowth of the organisms able to use molinate as a source of carbon as have been reported for other pollutants (Coppotelli et al. 2008; Wang et al. 2006). In fact, the initial molinate concentration in soil was negligible when compared with the amount of organic carbon; therefore, it could hardly be responsible for an observable increase in the cell number in these soils. A so called "microcosm effect" was also observed through the DGGE patterns and actinobacterial clone libraries as time course variations were observed not only in M and Mi microcosms but also in the controls (*C* and Ci). These results indicate the influence of the experimental design in the soil total and cultivable microbial communities.

The analysis of the DGGE patterns of the microcosms supported some conclusions on the effect of molinate on the soil total bacterial communities. The structure of the bacterial communities at day 0 in the control microcosms without molinate (*C* and Ci) and treatment microcosms (M and Mi) was slightly different, suggesting that the time (~2 h) taken to prepare the microcosms (between molinate

homogenization in soil and sampling at day 0) was enough to induce slight variations in the soil bacterial community. However, after 42 days of incubation, all the DGGE patterns were similar (M, Mi, *C* and Ci), suggesting that the effect of molinate in the bacterial community was transient. Indeed other authors reported that the impact of a given pollutant on the bacterial composition may only be transient. For example, structural shifts in the bacterial communities of soils spiked with either 2,4-dichlorophenoxyacetic acid or paenimyxin occurred only in the first days of exposure as at the end of the experiments no significant differences were observed between the bacterial communities from the assays and controls (Gonod et al. 2006; Selim et al. 2007). The transitory effect of molinate and other pesticides on soil microbial processes such as substrate-induced respiration and nitrification was also previously reported (Saison et al. 2009).

When bioaugmentation is used as a remediation strategy, the capacity of the exogenous microorganism(s) to degrade the pollutant without disturbances to the indigenous microbial populations is of major importance. However, inoculated microorganisms can overgrow and become dominant (Coppotelli et al. 2008; Gomes et al. 2005). Given that in the current study the exogenous microorganisms were able to survive and degrade the pollutant, it was important to assess possible disturbances in the Mi bacterial community. The analysis of the soil total bacterial communities (DGGE patterns) suggested that the inoculation with exogenous microorganisms did not affect the soil indigenous community. In fact, the DGGE patterns of Mi and of M microcosms clustered consistently. Additionally, none of the sequences retrieved from the actinobacterial libraries from the Mi and Ci microcosms could be affiliated to *G. molinativorax* ON4^{T}. Although it can be assumed that bias related with DNA extraction may have influenced this result, these results suggested that *G. molinativorax* ON4^{T} is present in low numbers in microcosms and may be outcompeted as a target for the modified group specific F243GM PCR primer and resulted in a higher amplification of the dominant microorganisms. In addition, the fact that the highest heterotrophic counts were found in the inoculated sterile soil with molinate (CsMi) suggests that the

exogenous culture is not capable of overgrowth in the presence of the indigenous microbiota.

The vast majority (87/119) of sequences from the treatment of M and Mi and controls (*C* and Ci) constructed actinobacterial libraries were affiliated to *S. shaanxiensis* CCNWHQ 0031^T, an organism isolated from soil in a sewage irrigation area (Lin et al. 2012). Organisms belonging to genus *Streptomyces* have been isolated and characterized as able to co-metabolize molinate (Daffonchio et al. 1999; Golovleva et al. 1981; Imai and Kuwatsuka 1986a; Imai and Kuwatsuka 1986b). The dominance of members of genus *Streptomyces* in the studied microcosms was not unexpected given that these are described as common soil inhabitants. Additionally, *in silico* analysis indicated that the highest number of 16S rRNA gene sequences matching primer F243GM was those of members of this genus (7,257 out of 9,165, assuming two sequence mismatches).

In summary, this study revealed that neither molinate nor the exogenous molinate mineralizing culture (mixed culture DC) induced significant perturbation of the total and cultivable bacterial communities in soil. Although natural attenuation may be used in the future to reduce molinate contamination in soil, a bioaugmentation strategy may be advantageous since a higher extent and/or rate of herbicide depletion was achieved when mixed culture DC was used as inoculum. Although being more extensive in the presence of mixed culture DC, molinate degradation also took place in non-inoculated microcosms. This observation, and the fact that *G. molinativorax* ON4^T or *mol*A gene could not be detected in non-inoculated microcosms, suggests that other modes of biodegradation may occur in soils. Indeed molinate hydrolase and members of species *G. molinativorax* are apparently rare and may have emerged in heavily contaminated sites (Barreiros et al. 2003) (http://www.ncbi.nlm.nih.gov), without further successful dissemination in the environment. Nevertheless, in mixed culture DC, the pivotal role is from *G. molinativorax* ON4^T. The auxiliary activity of the other four strains in mixed culture DC can, probably, be done in soils by autochthonous organisms. This

metabolic cooperation between *G. molinativorax* ON4^{T} and autochthonous organisms may explain the maintenance of this bacterium or of the *mol*A gene for long periods of time after inoculation.

Chapter 5

Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing

Results included in:

Lopes, A.R., Manaia, C.M. and Nunes, O.C. Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. (submitted for publication)

5.1 Abstract

Crop rotation is a practice harmonised with the sustainable rice production. Nevertheless, the implications of this empirical practice are not well characterized, mainly in relation to the bacterial community composition. This study addressed this topic, assessing the effect of the crop rotation stage, rice cycle and presence of the rice crop on the composition and structure of bacterial communities in soils in an alfalfa-rice rotation system. The bacterial communities of paddy fields in the 3rd and 4th year of the crop rotation cycle and of a non-seeded sub-plot were characterized over the rice cycle, using 454-pyrosequencing of 16S rRNA gene. Although the phyla *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes* predominated in all the samples, there were variations in the bacterial communities. Differences on the abundance of members of the phylum *Bacteroidetes* were observed at the successive stages of the crop rotation. Members of the phylum *Nitrospira* were more abundant after rice harvest. The presence of rice plants was positively correlated with members of the orders *Acidobacteriales* and "*Solibacterales*" and negatively with lineages such as *Chloroflexi* "Ellin6529". Studies like this represent important science-based tools to understand the influence of plants on soil microorganisms in sustainable rice production.

Keywords: 454-pyrosequencing, bacterial community, crop rotation, diversity, PCoA, rice paddy soil.

5.2 Introduction

Soil is a privileged habitat for microorganisms and is amongst the most biodiverse environments on Earth (Tamames et al. 2010). It is estimated that 1 gram of soil contains about 1 billion of prokaryotic cells (Roesch et al. 2007). Although the vast majority of microbial soil species are so far uncultivable, in the last decades, the development and improvement of culture-independent methods (e.g., fluorescent in *situ* hybridization-FISH, phyloarrays, fingerprinting techniques and sequencing of

small-insert libraries of environmental DNA) (Newby et al. 2009), allowed to get important insights into the phylogenetic diversity of soil microbiomes. Among them, high throughput sequencing technologies gave a new impetus to microbial ecology studies (Mardis 2008; Mardis 2011; Suenaga 2012). In spite of the biases inherent to these technologies (Suenaga 2012; Zinger et al. 2012), the pyrosequencing of phylogenetic marker genes, such as the 16S rRNA gene offers new insights into the composition and structure of environmental microbial communities, allowing inferences about biogeographical or ecological patterns in different habitats (e.g., soil, rhizosphere, sediments) (Baldrian et al. 2012; Gomes et al. 2010; Zhang et al. 2012) or environmental gradients (e.g., depth, pH, N amendments) (Eilers et al. 2012; Lauber et al. 2009; Ramirez et al. 2012).

Due to the increasing demand for productivity, in the beginning of the past century ancient agriculture practices gave way to conventional farming (Ladha and Reddy 2003; Matson et al. 1997). Despite the benefits on productivity, the use of synthetic chemical compounds strongly alters soil microbial communities composition and biogeochemical cycles (Hussain et al. 2009; Ramirez et al. 2012) and triggers strong negative impacts on sustainable soil fertility and on environmental quality (Galloway et al. 2008; Matson et al. 1997; Quayle et al. 2006). The expansion of sustainable agriculture practices, which avoid or strongly reduce the use of pesticides and synthetic fertilizers is a priority. Often relying on empirical practices, sustainable agriculture production would benefit from science-based evidences, demonstrating the benefits of such management practices.

Crop rotation is an important alternative to conventional farming, because it offers high productivity (Xuan et al., 2012) while contributes to environmental health (Rui and Zhang 2010). The cereal-legume rotation is a worldwide extended crop management, which improves the yield and quality of cereal crops by reducing diseases and weeds (Fenández-Aparicio et al. 2007; Liebman and Davis 2000), fixing atmospheric N_2 (Kelner et al. 1997), and contributing to increase the soil organic matter content (Rosen and Allan 2007). The legumes used in these rotation systems depend on the world region, the water regime and the season where forage

is being cropped (Ladha and Reddy 2003). Alfalfa (*Medicago sativa* L.) is a legume used in different world regions and with different crops, which contributes to maintain soil organic carbon (SOC) and to the accumulation of N in soil (Kelner et al. 1997; Pietsch et al. 2007). Nevertheless information about the effect of alfalfa-rice rotation system on the bulk soil bacterial community is scarce. The current study was designed for comparing the composition and structure of the bacterial communities in rice fields in different stages of an alfalfa-rice rotation system. In order to have a thorough perspective of these communities, 16S rRNA metagenome analyses were conducted using 454-pyrosequencing. Specifically it was intended to i) characterize the bulk soil bacterial community; ii) assess the influence of the crop rotation stage on the bacterial community structure; iii) identify the bacterial lineages with the highest and lowest vulnerability to changes over the rice crop cycle; iv) identify the lineages affected by the presence of rice plants.

5.3 Materials and Methods

5.3.1 Site description and soil sampling

The soil samples were collected in an experimental farm ("Bico da Barca", 40 º 11' N; 08 º 41' W), in the valley of river Mondego, Montemor-o-Velho, Central Portugal. Samples were collected from two adjacent organically farmed paddy fields, both under a 4 years crop rotation system, in which alfalfa (forage crop) rotates with rice (cereal crop). Briefly, alfalfa is cropped for two consecutive years (designated 1st and 2nd year) followed by two consecutive years of rice cropping (designated 3rd and 4th year). During rice cropping land is under fallow in autumn and winter to avoid the spreading of plant diseases and weeds (Fig. 5.1). At the beginning of the study paddies A and B were, respectively, at the 3rd and 4th year of the crop rotation cycle. In both paddies, the organic agricultural management of rice crop was similar to that described before (Lopes et al. 2011) with few exceptions; in April, one month before rice seeding, paddy B (but not paddy A) was amended with a plant residue commercial compost (Fertiormont, 2 t ha^{-1}) and

with mineral gafsa (Fertigafsa, 300 kg P ha^{-1}). These amendments intend to overcome the usual reduction of rice yield from the first to the second year of production.

Triplicate composite samples (I, II, III) each consisting of 20 individual soil cores pooled together were randomly collected from the upper 0-25 cm of the soil at paddies A (total area=3070 m^2) and B (total area=1715 m^2). Soils were sampled before seeding in early April 2010 (samples labelled A_{Apr} and B_{Apr}, respectively) and after harvesting in late September (A_{Sep} and B_{Sep}, respectively). To assess the effect of the rice plants on the microbial community of the bulk soil, in September composite samples were collected from a non-seeded sub-plot of paddy A (ANS_{Sep}, non-seeded). After removal of visible root debris and homogenization, soil aliquots of each replica were immediately stored at 4 ºC for soil physicochemical characterization and at -20 ºC for molecular characterization.

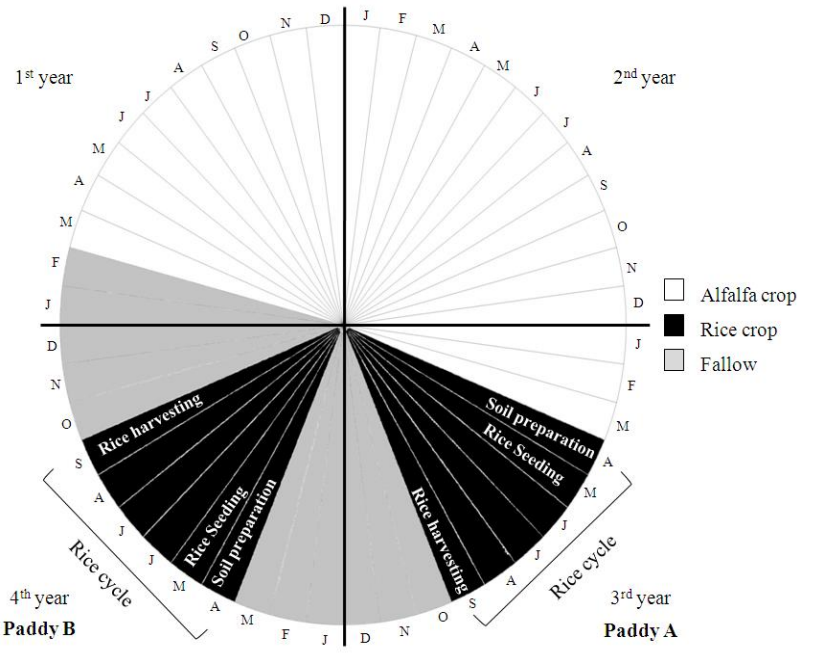

Fig. 5.1 Scheme of alfalfa-rice crop rotation in the experimental farm "Bico da Barca", Montemor-o-Velho, Portugal.

5.3.2 Soil characteristics

The total C and N contents (wet oxidation with dichromate and Kjeldahl digestion, respectively), pH in water and water content were determined as described by Guitián and Carballas (1976) . Total available-P was extracted with 0.5 M sodium bicarbonate (pH 8.2) (Bowman and Cole 1978). All analyses were performed on triplicate soil samples.

5.3.3 16S rRNA gene barcode 454-pyrosequencing

A barcode pyrosequencing approach was used for the characterization of soil bacterial communities. Genomic DNA was extracted from 7 aliquots of each soil replica using the Power Soil™ DNA Isolation Kit (MO BIO) as described before (Lopes et al. 2011), collecting the seven extracts in a single tube. DNA was further purified (Bacteria genomicPrep Mini Spin Kit, Amersham Biosciences, NJ, USA). The DNA concentration in the final extracts (Qubit® Fluorometer (Invitrogen) with Quant-iT™ dsDNA HS assay kit) was approximately 20 μg ml^{-1}. DNA extracts were used as template for the amplification by PCR of the hypervariable V3-V4 region (~360 bp) of the 16S rRNA gene. The PCR amplifications, performed in duplicate for each DNA extract, were carried out using barcoded fusion primers containing the Roche-454 A and B Titanium sequencing adapters, an eight-base barcode sequence in adaptor A, and specific sequences for the ribosomal region (V3F 5'-ACTCCTACGGGAGGCAG-3' and V4R 5'-TACNVRRGTHTCTAATYC-3') (Wang and Qian 2009). PCR mixtures (25 μl) contained: 0.2 mM dNTPs (Bioron, Ludwigshafen am Rhein, Germany), 0.2 μM of each primer, 5 % DMSO (Roche Diagnostics GmbH, Mannheim, Germany), 1x Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA), 1x Advantage 2 PCR Buffer, and 1-3 μL of target DNA, and cycling conditions consisted of a first denaturation step at 94 °C for 4 min, followed by 20 cycles at 94 °C (30 s), 44 °C (45 s) and 68 °C (60 s), and a final 2 min extension at 68 °C. The amplicons were quantified by fluorimetry with PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA) and pooled at

equimolar concentrations. Pyrosequencing libraries were obtained using the 454 Genome Sequencer FLX platform according to standard 454 protocols (Roche 454 Life Sciences, Brandford, CT, USA) at Biocant (Cantanhede, Portugal). The raw reads have been deposited into the NCBI short-reads archive database (accession number: SAMN01908502 to SAMN01908516).

5.3.4 Post-run analysis

Data generated from pyrosequencing was processed and analysed using QIIME pipeline (Caporaso et al. 2010). Briefly, sequences shorter than 280 bp and with quality scores lower than 25 were eliminated. Sequences (> 280 bp) were assigned to samples by the 8-bp barcodes and grouped into operational taxonomic units (OTUs) using uclust (Edgar 2010) with a phylotype threshold of ≥ 97 % sequence similarity. Representative sequences were taxonomically assigned, using QIIME defaults (5-level assignment). Representative sequences were aligned using PyNAST (DeSantis et al. 2006) and were classified using Ribosomal Database Project (RDP) classifier (Wang et al. 2007). At the 97 % identity level, the final OTU table consisted of 67 566 sequences (average of 4504 sequences per replica) distributed into 9480 OTUs, of those 4444 were represented by more than one sequence. A phylogenetic tree containing the aligned sequences was produced using FastTree (Price et al. 2009).

Both alpha and beta diversity metrics were determined using the QIIME pipeline. Alpha diversity was assessed calculating Chao 1, Simpson (Simpson 1949), Shannon (Shannon and Weaver 1963) and phylogenetic diversity (PD) (Faith 1992) metrics. Additionally, beta diversity patterns of rarefied samples (2700 sequences per replica) were assessed using the UniFrac metric (Lozupone and Knight 2005).

5.3.5 Statistical analysis

The soil physicochemical properties and alpha diversity metrics among the five soil samples ($A_{Apr/Sep}$, ANS_{Sep} and $B_{Apr/Sep}$) were compared using the two way analysis of variance (ANOVA) and the post-hoc Tukey test (SPSS Statistics 19, IBM).

Taxon and phylogenetic-based analyses were used to compare 16S rRNA gene sequences among the soil samples and to identify the bacterial lineages that significantly changed over i) crop rotation, ii) rice crop cycle and iii) that were affected by the presence of rice plants. For the taxon-based analyses, the percentage of abundance OTUs were compared using ANOVA combined with post hoc Tukey test. Phylogenetic-based comparisons were done using QIIME pipeline. Jackknifed principal coordinate analysis (PCoA) and dendrograms based on (un)weighted UniFrac distances were obtained. Statistical differences between bacterial communities from the studied samples plotted on both PCoA were tested using analysis of similarity (ANOSIM). A Mantel test was conducted to evaluate if any of the determined soil physicochemical properties were related to the (un)weighted UniFrac values determined for soil samples and plotted on the PCoA.

5.4 Results

5.4.1 Physicochemical properties

The soils analyses showed similar physicochemical characteristics although some significant differences in pH, water content, total nitrogen and concentration of labile phosphorous among the five samples were found (Table 5.1). The total carbon content was not significantly different in the analysed samples.

Table 5.1 Physicochemical properties of soils A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. Values are means ± standard deviation (n=3).

Parameter	A_{Apr}	A_{Sep}	ANS_{Sep}	B_{Apr}	B_{Sep}
pH in water	5.97 ± 0.03 ^{C}	5.88 ± 0.02 ^{B}	5.87 ± 0.02 ^{B}	5.94 ± 0.06 ^{B,C}	5.70 ± 0.02 ^{A}
Water content (%) (g H_2O/100 g wet soil)	22.4 ± 0.1 ^{B,C}	21.8 ± 0.4 ^{B}	19.2 ± 0.5 ^{A}	27.1 ± 0.8 ^{D}	23.4 ± 0.2 ^{C}
Total C (%)	1.33 ± 0.10	1.33 ± 0.13	1.38 ± 0.27	1.68 ± 0.04	1.60 ± 0.05
Total N (%)	0.14 ± 0.00 ^{A}	0.14 ± 0.00 ^{A}	0.14 ± 0.00 ^{A}	0.15 ± 0.01 ^{A}	0.18 ± 0.00 ^{B}
Total available-P (mg P kg^{-1})	50.7 ± 0.4 ^{B}	35.7 ± 0.6 ^{A}	36.7 ± 0.6 ^{A}	80.3 ± 2.4 ^{D}	56.4 ± 0.6 ^{C}

The typical texture of this soil is about 28, 50 and 21 % of sand, silt and clay, respectively (Lopes et al. 2011). The rice productivity was higher in paddy A than in paddy B (productivity ratio B:A = 0.63). A-D, Homogeneous subsets among paddies (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}), as determined by the Tukey test at $P<0.05$.

5.4.2 Bacterial diversity

In total, 67 566 high-quality sequences from the fifteen analysed metagenomes (triplicate of the samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}) were obtained. Approximately 2 % of these sequences were not affiliated to the domain *Bacteria* and were excluded from further analyses. A variable number of high-quality sequences was obtained for replicas and samples (lowest and highest number of sequences were 2754 and 7023, respectively); for this reason replicas were normalized by rarefaction to 2700 sequences per replica (total of 8100 sequences per soil sample).

Similar values of estimator Chao1 and Simpson index were obtained for all samples, demonstrating, respectively, similar diversity coverage and an even distribution of sequences in the studied samples. The number of OTUs per rarefied soil sample varied between 1170 (B_{Sep}) and 1290 ($ANSs_{ep}$). Accordingly, sample ANS_{Sep} showed the highest PD and Shannon index values ($P < 0.05$) (Table 5.2), which indicates that the bacterial community in this soil was more diverse than in cropped soils ($A_{Apr/}s_{ep}$ and $B_{Apr/Sep}$).

Table 5.2 Diversity of bacterial rRNA gene fragment sequences in soil samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. The diversity measurements (Number of OTUs, Chao1, Shannon and Simpson indices and Phylogenetic diversity) were determined at a rarefaction of 2700 sequences per replica. Values are means ± standard deviation (n =3).

	A_{Apr}	A_{Sep}	ANS_{Sep}	B_{Apr}	B_{Sep}
No. OTUs	1236 ± 12 ^{A,B}	1206 ± 41 ^{A,B}	1290 ± 45 ^{B}	1212 ± 40 ^{A,B}	1170 ± 29 ^{A}
Chao1	3150 ± 114	2967 ± 148	2967 ± 184	3060 ± 163	3010 ± 36
Shannon index	9.30 ± 0.02 ^{B,C}	9.26 ± 0.07 ^{A,B,C}	9.44 ± 0.08 ^{C}	9.25 ± 0.03 ^{A,B}	9.12 ± 0.03 ^{A}
Simpson index	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
Phylogenetic Diversity (PD)	87.2 ± 1.2 ^{A}	89.7 ± 2.8 ^{A}	99.2 ± 4.1 ^{B}	90.5 ± 2.7 ^{A,B}	88.2 ± 1.7 ^{A}

A-C, different letters indicate differences in alpha diversity metrics values among the bacterial communities of the analysed samples (one-way ANOVA followed by Tukey post-hoc test, $P < 0.05$).

5.4.3 Taxonomic affiliation of the total rarefied sequences

Each analysed metagenome included sequences that could not be assigned beyond the bacterial domain (average of 2.2 ± 0.3 %). The remaining bacterial sequences were affiliated to 39 phyla, although only 19 had sequence abundances above 0.1 % (Fig. 5.2 and Table S5.1). Among these 19 phyla, sequences classified as *Acidobacteria* (32.4 %), *Proteobacteria* (26.3 %), *Chloroflexi* (8.6 %), *Actinobacteria* (7.5 %), *Bacteroidetes* (7.3 %), and *Gemmatimonadetes* (6.6 %) comprised about 80–90 % of all sequences. Other phyla with average abundance >1 % included *Nitrospira* (2.7 %), *Elusimicrobia* and *Firmicutes* (both averaging 1.0 %). The phyla *Chlorobi*, *Cyanobacteria*, *Verrucomicrobia*, *Planctomycetes* and candidate phyla, such as TM7 and AD3, among others were less abundant (abundance ranging from 0.1 to 1 %) (Fig. 5.2).

Acidobacteria, the most abundant phylum, was represented mainly by sequences affiliated to the classes *Acidobacteria* (16.0 %, out of which 99 % belonged to *"Koribacteraceae"*), and "*Solibacteres*" (11.0 %, all included in "*Solibacteraceae*"). Most of the *Proteobacteria* sequences were classified as

Alphaproteobacteria (23.3 %, out of which 45 % belonged to *Sphingomonadaceae*) (Table S5.2). Thus, these three were the most abundant families in the analysed metagenomes, representing 26–43 % of the total of sequences (Fig. S5.1 and Table S5.4).

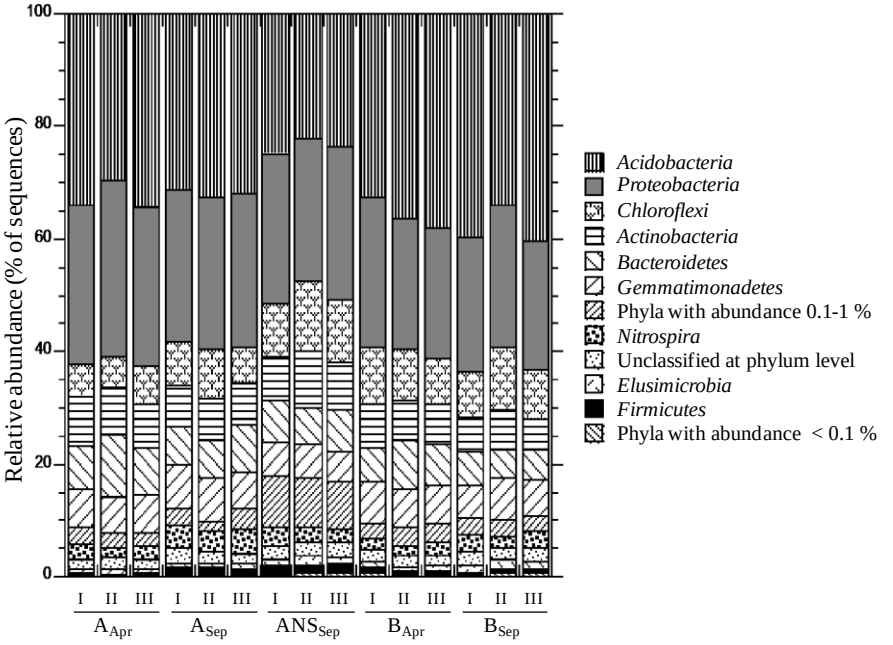

Fig. 5.2 Relative abundance of different phyla in each replica of samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. The abundance is expressed as the percentage in the total number of rarified bacterial sequences (2700), classified using RDP classifier at a confidence threshold of 80%. Phyla with abundance ranging from 0.1 to 1% include *Chlorobi*, *Cyanobacteria*, TM7, AD3, OD1, *Armatimonadetes*, *Verrucomicrobia*, *Fibrobacteres*, *Planctomycetes* and OP11. Phyla with abundance < 0.1% are listed in the Table S5.1.

5.4.4 OTUs distribution and taxonomic affiliation

Considering the nucleotide sequence similarity threshold value of ≥ 97 %, a total of 6748 OTUs were obtained from rarefied data of the five soil samples analysed. Of these OTUs, 470 were common to all samples, 601 were common to the cropped

samples ($A_{Apr/Sep}$,B_{Apr} and B_{Sep}), and 688 were common to samples A, irrespective of rice plant presence ($A_{Apr/Sep}$ and $ANSs_{ep}$) (Figs S5.2a and b).

Taking into account only OTUs that occurred with an abundance > 0.1 % in at least one sample (herein considered representative OTUs) the value of 6748 was reduced to 371. Of these, 276 were common to all samples, 297 were common to samples $A_{Apr/Sep}$ and $B_{Apr/Sep}$ and 299 were common to samples A, irrespective of rice plant presence ($A_{Apr/Sep}$ and $ANSs_{ep}$). Thus, about 80 % of the representative OTUs were common to all samples and unique OTUs were, in general, rare (less than 0.1 % abundance). Regardless the threshold of abundance used to create Venn diagrams, sample $ANSs_{ep}$ showed the highest number of unique OTUs (Fig. S5.2).

Among the 276 OTUs common to the five samples, only 82 had abundance > 0.1 % in all the samples, being considered core OTUs. These OTUs were affiliated to the most abundant phyla, except one that was assigned to the candidate phylum AD3 (Table 5.3). As could be expected, the majority of core OTUs were evenly distributed, presenting abundances between 0.1 and 1 %. Exceptions were two OTUs assigned to the families *Sphingomonadaceae* and "*Koribacteraceae*", which represented, in average, 6.9 and 1.3 % of the total recovered diversity (Table 5.3 and Fig. S5.1).

Table 5.3 Number of core OTUs (abundance > 0.1 %) in the five samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}.

Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
OTUs present in all the samples at abundance > 1 %									
Bacteria	2	*Acidobacteria*	1	*Acidobacteria*	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
		Proteobacteria	1	*Alphaproteobacteria*	1	*Sphingomonadales*	1	*Sphingomonadaceae*	1
OTUs present in all the samples with abundances ranging from 0.1 to 1%									
Bacteria	80	*Acidobacteria*	39	*Acidobacteria*	20	*Acidobacteriales*	20	"*Koribacteraceae*"	19
								Acidobacteriaceae	1
				"*Acidobacteria* - 2"	3				
				"*Chloroacidobacteria*"	3				
				"*Solibacteres*"	13	"*Solibacterales*"	13	"*Solibacteraceae*"	13
		Actinobacteria	3	*Acidimicrobiia*	1	*Acidimicrobiales*	1		
				Actinobacteria	2	*Actinomycetales*	2	*Intrasporangiaceae*	1
								Micrococcaceae	1
		AD3	1	"ABS-6"	1				
		Bacteroidetes	3	*Sphingobacteriia*	3	*Sphingobacteriales*	3	*Chitinophagaceae*	2
		Chloroflexi	1	"Ellin 6529"	1				
		Gemmatimonadetes	10	*Gemmatimonadetes*	6				
				"Gemm-1"	4				
		Nitrospira	2	*Nitrospira*	2	*Nitrospirales*	2	*Nitrospiraceae*	2

Table 5.3 Continued

Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
OTUs present in all the samples with abundances ranging from 0.1 to 1%									
Bacteria	80	*Proteobacteria*	20	*Alphaproteobacteria*	19	*Rhizobiales*	7	*Beijerinckiaceae*	1
								Bradyrhizobiaceae	1
								Hyphomicrobiaceae	4
								Phyllobacteriaceae	1
						Caulobacterales	1	*Caulobacteraceae*	1
						Rhodospirillales	3	*Rhodospirillaceae*	3
						Sphingomonadales	6	*Sphingomonadaceae*	6
				Deltaproteobacteria	1	*Myxococcales*	1	*Cystobacteriaceae*	1

5.4.5 Relationship between bacterial communities and edaphic factors

To compare the composition and structure of the bacterial communities of the analysed soil samples, the beta diversity was assessed. The unweighted and weighted UniFrac-based PCoA explained, respectively, 21.6 and 55.9 % of the total variation among the structure and composition of the bacterial communities, and supported the distribution of samples in distinct groups (Figs 5.3a and b). ANOSIM confirmed that the groups plotted in both PCoA were significantly different (unweighted UniFrac: R= 0.97, *P* = 0.001; weighted UniFrac: R= 0.89, *P* = 0.001). Bacterial lineages affiliated to abundant phyla (Fig. 5.2) contributed to the variations observed in the weighted UniFrac PCoA (Figs 5.3b and c). Correlations between UniFrac values and edaphic parameters were determined using Mantel tests (Table 5.4). A mild correlation between unweighted distances and water, total carbon and labile phosphorous contents, and C:P and N:P ratios was observed. On the other hand, a mild correlation between weighted distances and water and total carbon contents was observed.

Table 5.4 Relationship of soil physicochemical properties and (un)weighted unifrac distance measured from soil samples (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}) (Mantel test).

Soil physicochemical properties	Unweighted		Weighted	
	Mantel *r*	*P* value	Mantel *r*	*P* value
Water content	0.527	0.001**	0.406	0.004*
pH	0.095	0.390	0.055	0.670
Total C	0.230	0.039*	0.271	0.036*
Total N	0.033	0.765	0.138	0.372
Total available-P	0.347	0.003*	0.133	0.290
C:N	0.168	0.100	0.120	0.301
C:P	0.242	0.022*	0.162	0.195
N:P	0.324	0.016*	0.076	0.505

The iterations were set to 999. *$P < 0.05$ and ** $P \leq 0.001$ indicate significant correlations.

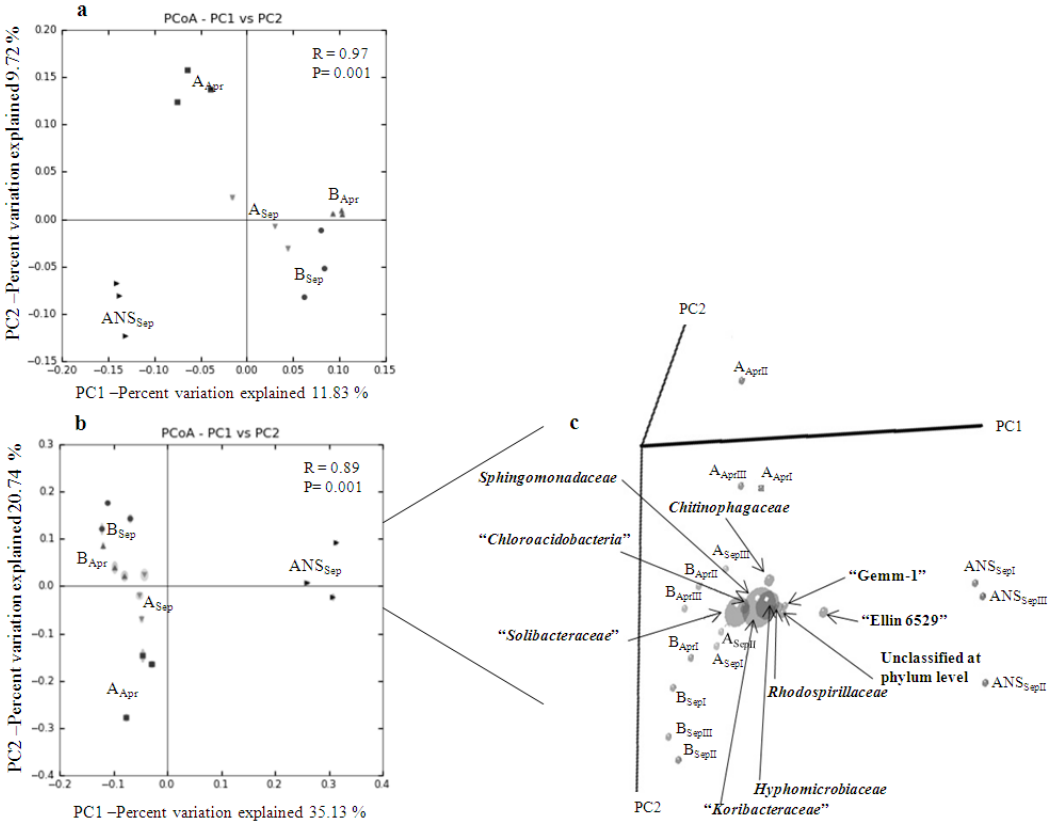

Fig. 5.3 Jackknifed PCoA plots illustrating distances among bacterial communities of soil samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. **a)** Bacterial unweighted UniFrac distances. **b)** Bacterial weighted UniFrac distances. **c)** Bacterial lineages that varied most among samples.

5.4.6 Effect of *Oriza sativa*

A strong primary clustering of $A_{Apr/Sep}$, B_{Apr} and B_{Sep} *versus* $ANSs_{ep}$ soil samples, represented in both UniFrac-based PCoA biplots (Figs 5.3a and b), evidenced differences between the bacterial communities of cropped and uncropped soil samples. In fact, the average UniFrac distances measured between uncropped ($ANSs_{ep}$) and cropped soils samples ($A_{Apr/Sep}$ or $B_{Apr/Sep}$) were the highest (Fig. S5.4). Several representative OTUs in cropped samples, such as those assigned to "*Solibacteraceae*", "*Koriobacteraceae*", *Sphingomonadaceae,* were absent or rare in the uncropped soil sample. Inversely, several representative OTUs in sample ANS_{Sep}, affiliated to *Chloroflexi* ("Ellin 6529"), "*Acidobacteria*-2", AD3, *Nostocaceae,* among others, were absent or rare in samples $A_{Apr/Sep}$, B_{Apr} and B_{Sep} (Table S5.5). The majority of these OTUs were affiliated to bacterial lineages that correlated most with the different groups depicted in the weighted PCoA (Fig. 5.3b and c). Also the higher abundance of unclassified sequences in ANS_{Sep} than in the other samples contributed to distinguish its bacterial community from the others.

5.4.7 Effect of crop rotation

Differences among the bacterial communities of soil samples at different stages of crop rotation were plotted in both UniFrac-based PCoA (Figs 5.3a and b). These analyses showed that the bacterial communities from the 3rd ($A_{Apr/Sep}$) and 4th year ($B_{Apr/Sep}$) cropped soil samples did not cluster together (Figs 5.3a and b, and S5.3a and b). A gradient of variation from A_{Apr} to B_{Sep} along axis 2 is shown in the weighted UniFrac-based PCoA biplot (Fig. 5.3b). The highest and lowest average UniFrac distance between each pair of these samples was found, respectively, for the pairs A_{Apr}/Bs_{ep} and $B_{Apr/Sep}$ (Figs S5.4a and b). A higher similarity between the bacterial communities of soil B sampled in different periods of the rice cycle than with samples of soil A is evidenced. However, the bacterial community of sample

A_{Sep} was closer to samples of the 4th year of crop rotation ($B_{Apr/Sep,}$ mainly to B_{Apr}) than to A_{Apr}.

Sample A_{Apr} contained a higher number of rare and representative unique OTUs than the other cropped soil samples (Fig. S5.2). Representative unique OTUs assigned to *Actinobacteria* (*Cellulomonadaceae*) and *Bacteroidetes* (*Sphingobacteriales*, mainly of the family *Chitinophagaceae*) (Table S6) present in A_{Apr} made this sample the most distinct amongst the cropped soil samples (Figs 5.3b and c).

The distinction of the bacterial communities over the crop rotation stage was supported by some OTUs whose abundance decreased [e.g., assigned to *Bacteroidetes* (*Sphingobacteriales*, *Flavobacteriales)*, and *Alphaproteobacteria* (*Caulobacteraceae*)] or increased [e.g., assigned to *Acidobacteria* (*Acidobacteriales*, "*Solibacterales*"), *Bacteroidetes* (*Bacteroidales*), *Chloroflexi* (*Anaerolineae*) and *Chlorobi* ("SJA-28")] from the 3rd year ($A_{Apr/Sep}$) to the 4th year ($B_{Apr/Sep}$) of crop rotation (Table S5.6).

5.4.8 Effect of rice cycle (seasonal effect)

The average UniFrac distances obtained between cropped soil samples collected in April and September in the 3rd ($A_{Apr/Sep}$) or in the 4th year ($B_{Apr/Sep}$) (Figs S5.4a and b) indicate variations, although small, of the bacterial communities over the rice cycle, mainly in soil A. Unweighted, but not weighted, UniFrac clustering via UPGMA showed some overlapping in the composition of 4th year (B) bacterial communities of April and September (Fig. S5.3a). It is, thus, suggested that major variations in the bacterial community over this period were mainly due to modifications on the relative abundance of soil bacterial members. OTUs assigned to "*Thermodesulfovibrionaceae*" within the phylum *Nitrospira*, which were more abundant in September than in April in both cropped soils (A and B), may have contributed to the observed variation (Table S5.7).

5.5 Discussion

5.5.1 Structure and composition of bulk soil bacterial community

The predominance of *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes* in the paddy soils studied was expected, since these groups have been described as common inhabitants of agriculture soils, including rice paddy soils (Asakawa and Kimura 2008; Tanahashi et al. 2005; Xuan et al. 2012). Half of the 82 core OTUs identified in this study belonged to the classes *Acidobacteria* and *Alphaproteobacteria*. *Alphaproteobacteria* comprise organisms with distinct physiological properties (Rappé and Giovannoni 2003), limiting a clear prediction about the ecology and biochemical role of these members in that habitat. However, it is noted that *Alphaproteobacteria* related with core OTUs enclose chemoorganotrophs, some of which are catalase producers and may be involved in lignin (e.g., *Caulobacter*) (Steinman et al. 1997) or aromatic compounds degradation (e.g., *Sphingomonadaceae*) (Kersters et al. 2006). Nitrogen fixation is another relevant function found in (photo)organotrophic *Alphaproteobacteria* (e.g., *Rhodospirillaceae, Hyphomicrobiaceae*) (Boer et al. 2005; DeAngelis et al. 2011; Heising and Schink 1998; Kersters et al. 2006). In comparison to *Alphaproteobacteria*, *Acidobacteria* and *Gemmatimonadetes* are poorly characterized, since only a small fraction of these bacteria were cultivated (Eichorst et al. 2007; Kishimoto et al. 1991; Zhang et al. 2003). Nevertheless, *Gemmatimonadetes* represents a phylum with ubiquitous distribution (DeBruyn et al. 2011; Rappé and Giovannoni 2003). *Acidobacteria* are common soil inhabitants, which abundance has been strongly correlated with pH gradients (Jones et al. 2009) and low C availability (Fierer et al. 2007a). The edaphic characteristics of the analysed soils seem to have favoured the abundance of members of this phylum, mainly of the families "*Koriobacteraceae*" and "*Solibacteraceae*". Denitrification, iron scavenging as well as decomposition of complex substrates (e.g., xylan, hemicellulose) are predicted functions of acidobacterial members (Ward et al. 2009). Altogether, these results suggest that

core bacterial members may have an important role in the cycling of plant debris through the decomposition of complex substrates, and to sustain metabolic transformations in the N and Fe biogeochemical cycles, which are crucial activities in paddy soils (Mårtensson et al. 2009; Ratering and Schnell 2000; Rui et al. 2009).

Verrucomicrobia have been described as abundant inhabitants of paddy soils (Asakawa and Kimura 2008; Kikuchi et al. 2007; Xuan et al. 2012), although presented low abundance in the current study. This observation may be due to the primers used, since *in silico* analysis demonstrated that these primer set may fail the detection of verrucomicrobial sequences (Wang and Qian 2009), explaining the underestimation of this phylum by culture independent methods (Bergmann et al. 2011).

5.5.2 Variations in the structure and composition of bulk soil bacterial community

The present study compared the composition and structure of the soil bacterial community as a function of the rice cycle, the crop rotation stage, and the presence of the rice crop. The presence of rice plants was the factor that affected most the bacterial diversity and community structure of bulk soil. In fact, alpha and beta diversity analyses revealed that the bacterial community of uncropped soil (ANS_{Sep}) was more diverse than those of cropped soil samples ($A_{Apr/Sep}$ and $B_{Apr/Sep}$). The bacterial communities of rhizosphere are known to be less diverse than those of bulk soil (Sørensen 1997). On the other hand, the type of plants influences the diversity, composition and structure of bulk soil bacterial communities (Xuan et al., 2012). Thus, the roots/rhizosphere effect may explain the differences observed in the present study. The dense root mat characteristic of rice cropped paddy soils (Liesack et al. 2000), combined with the usual flooding state of paddy fields may promote the distribution of root exudates in flooded soils. The presence of rice root exudates may have favoured the development of

organisms affiliated to "*Solibacteraceae*", "*Koriobacteraceae*" and *Sphingobacteraceae*, the most abundant bacterial lineages of the studied cropped soils. The proliferation of the organisms of these lineages may have contributed to lessen others, such as unclassified bacteria and members of "*Acidobacteria*-2" and AD3, absent or rare in cropped soils. In contrast, the absence of rice rhizosphere with consequent low amounts of root exudates in the uncropped soil could have favoured the development of bacterial lineages with slow growth and ability to survive under low substrate availability (e.g., *Chloroflexi* "Ellin6529"), as was described before for members of this phylum (Acosta-Martínez et al. 2010). Although constituting a sub-plot of paddy A, and thus, under the same flood regime, the water content in the uncropped soil was significantly lower than in cropped paddies. This difference was probably due to the ability of plants to increase the retention of water in soil (Chapin and Körner 1995). The fact that *Nostoc* members have been referred to be more prevalent in dry than in wet paddy soils (Roger et al. 1993) may explain the higher abundance of OTUs affiliated to *Nostocaceae* in uncropped than in cropped soil samples. On the other hand, the low plant cover of the uncropped sub-plot may have also favoured these photosynthesizing microorganisms.

The stage of the crop rotation affected the bacterial diversity and community structure of cropped bulk soil. Through the weighted UniFrac analysis, a temporal gradient from A_{Apr} to B_{Sep} was observed. The high number of rare and representative unique OTUs (*Bacteroidetes/ Sphingobacteriales* and *Actinobacteria/Cellulomonadaceae*) together with the abundance of other presumably aerobic chemoorganotrophs (*Bacteroidetes*/*Flavobacteriales* and *Alphaproteobacteria/Caulobacteraceae)* in sample $A_{Apr,}$ contributed to distinguish this sample from the others. Given that sample A_{Apr} was collected soon after alfalfa harvesting, the root exudates and debris of the forage crop may have favored the development of these bacterial lineages. A decrease in the abundance of these lineages with the simultaneous increase of others assigned to the most abundant acidobacterial orders (*Acidobacteriales* and "*Solibacterales*") over time, *i.e.*, from

A_{Apr} to B_{Set} occurred (Table S5.3). This finding suggests that the substitution of alfalfa debris for rice root exudates/debris promoted the development of *Acidobacteria* members, an effect detected also when comparing the rice cropped and uncropped soils.

Because "Bico da Barca" farmers observed before a sharp decrease on the rice productivity between the 3rd and 4th year of the alfalfa-rice rotation, they amended the soil. This was the reason for the mineral P and compost amendments made in paddy B, before rice seeding, which increased significantly the labile-P content of samples B. The increase in the abundance of presumably obligate anaerobic chemoorganotrophs (*Chloroflexi/Anaerolineae* and *Bacteroidetes/Bacteroidales)* and phototrophs (*Chlorobi* "SJA-28") from the 3rd to the 4th year of the crop rotation was observed. Beside the changes in the available nutrients, derived from the substitution of alfalfa for rice root exudates/debris and from the soil amendments, also the higher water content of samples $B_{Apr/Sep}$ than in $A_{Apr/Sep}$ may have contributed to the increased abundance of the presumably anaerobic members in the 4th year of crop rotation. It is worth mentioning that despite the amendments made, the rice productivity decreased from the 3rd to the 4th year of the crop rotation.

According to the results obtained, variations on the bulk soil bacterial communities over the rice cycle (seasonal variations) were mainly due to alterations in the abundance of some bacterial lineages rather than to presence/absence variations. Similar findings were reported in previous studies based on community analyses fingerprinting (Kikuchi et al. 2007; Lopes et al. 2011). Among the representative bacterial lineages occurring in the studied soils, members affiliated to "*Thermodesulfovibrionaceae*", within *Nitrospira,* stood out as having higher abundance in cropped soils after rice harvesting (A_{Sep} and B_{Sep}) than before rice seeding. The increased abundance of this phylum after rice harvesting was reported before (Xuan et al. 2012), and may be due to the availability of inorganic compounds resultant from organotrophic metabolism of plant debris and root

exudates. Indeed, *Nitrospira* includes chemolithotrophs involved on the N, Fe and S cycles through the activity of nitrite- and ferrous iron-oxidizing and sulfate-reducing bacteria (Ehrich et al. 1995; Henry et al. 1994; Hippe 2000). The development of sulfate-reducing bacteria, most probably obligate anaerobes, may have been also favoured by the flooding conditions prevalent during the growth of rice plants. If these lineages become prevalent in the late stage of the growth of rice plants, their predominance may become evident after rice harvesting. Indeed, the activity of sulfate and iron reducers is important in paddy soils (Liesack et al. 2000), and can be observed in old rice roots, due to blackening by ferrous sulfide precipitation at the late stage of the growth of rice plants.

In summary, the present study revealed that the most abundant phyla in paddy bulk soil from the alfalfa-rice rotation were *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes*. Despite these similarities, the bacterial community composition was affected by the crop rotation stage, the rice cycle and the presence of rice crop. Rice plants stand out as being the major determinants of community structure in bulk soil. Uncropped soil had the most diverse bacterial community, suggesting that the absence of rice plant allows the proliferation of specific lineages that may be outcompeted in cropped soils. The rice crop cycle may favour the proliferation of members of the phylum *Nitrospira*, which prevail in bulk soil after harvest. The differences induced by crop rotation stage may have been related to the attenuation of the alfalfa effect, soil exhaustion and amendments. Bacteria play a pivotal role on the turnover of biological materials and minerals in soils. Studies elucidating the variations of the bacterial communities in agriculture soils represent a valuable science-based approach for promoting a sustainable agriculture practice.

5.6 Supplemental Material

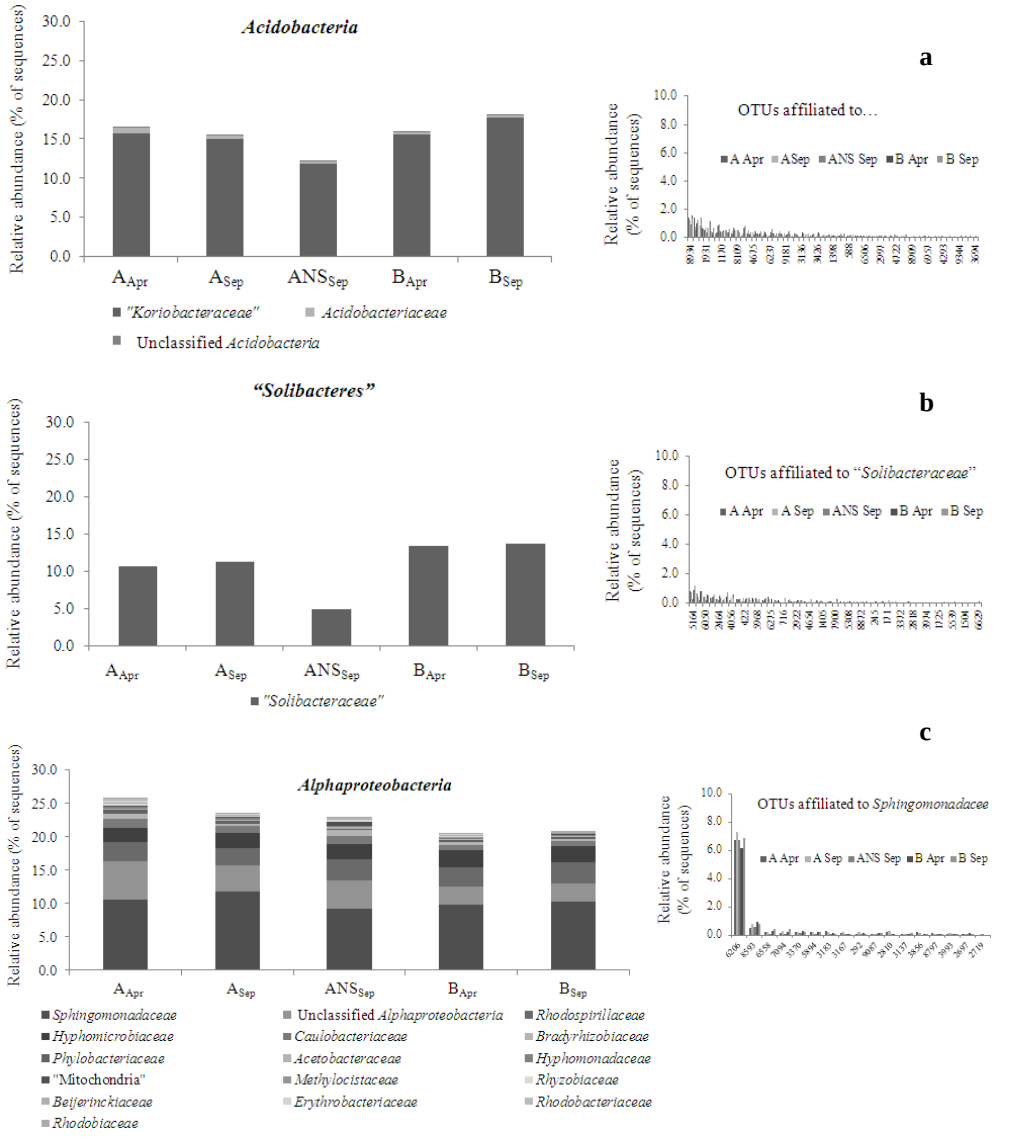

Fig. S 5.1 Relative abundance of families affiliated to the most abundant classes of *Acidobacteria* and *Proteobacteria* in samples (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}) and rank-abundance curves of OTUs (abundance > 0.1 %) affiliated to the most abundant family in each class. (a) *Acidobacteria* ("*Koribacteraceae*"). (b) "*Solibacteres*" ("*Solibacteraceae*"). (c) *Alphaproteobacteria* (*Spingomonadaceae*).

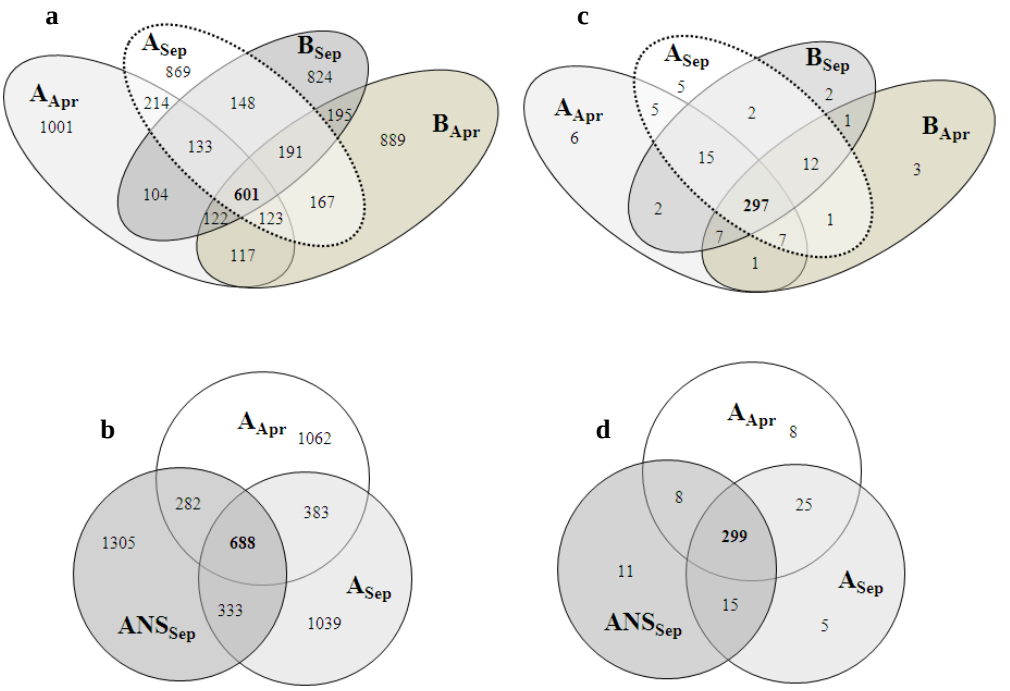

Fig. S 5.2 Venn diagrams showing the distribution of the OTUs that were unique or common among samples. **a)** All OTUs in samples $A_{Apr/Sep}$,B_{Apr} and B_{Sep}. **b)** All OTUs in samples $A_{Apr/Sep}$ and ANS_{Sep}. **c)** OTUs with abundance > 0.1 % in at least one sample ($A_{Apr/Sep}$,B_{Apr} and B_{Sep}). **d)** OTUs with abundance > 0.1 % in at least one sample ($A_{Apr/Sep}$ and ANS_{Sep}).

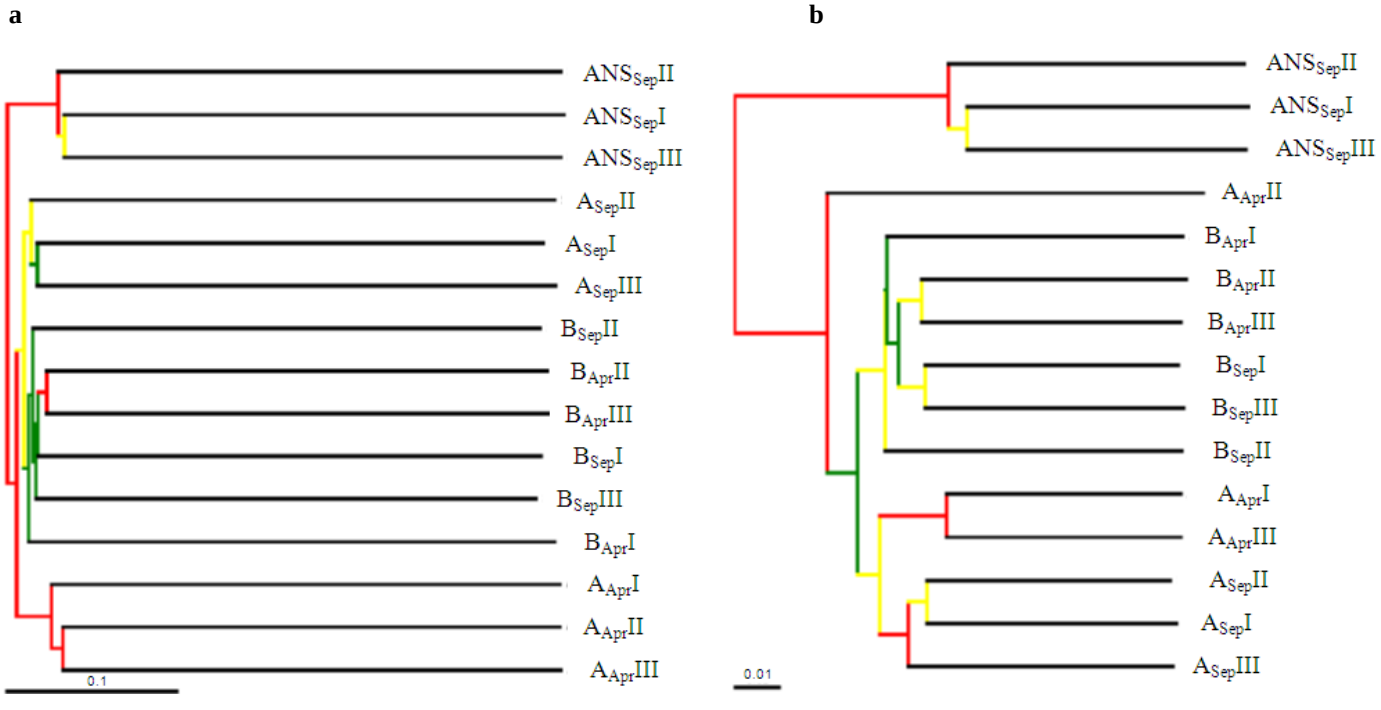

Fig. S 5.3 Dendrograms based on UPGMA clustering of rarefied data from soil samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}. Generated using the **a)** unweighted and **b)** the weighted UniFrac distances. The colored nodes on the left indicate the confidence levels that support each node: 75-100 % (red), 50-75 % (yellow) and 25-50 % (green).

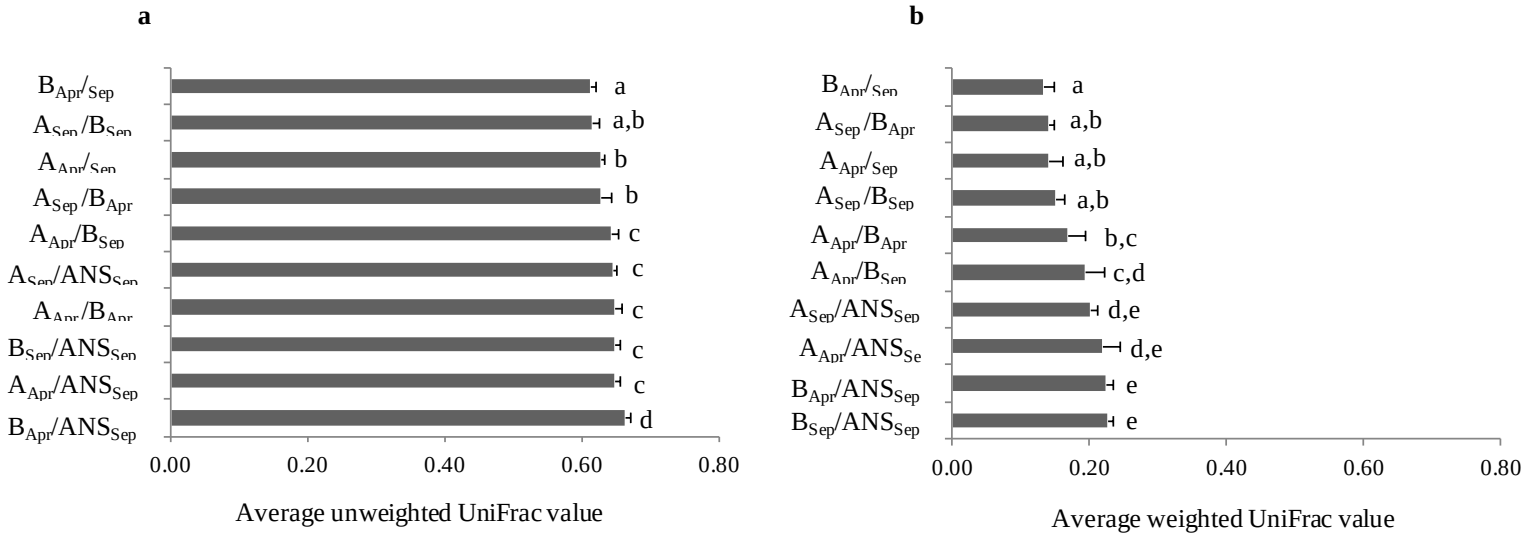

Fig. S 5.4 Variations in community structure between the studied samples as measured by the average UniFrac distance within each sample pair (y-axis). Error bars are the standard deviation of the UniFrac distances between pairs of replicas within each sample pair. **a)** unweighted. **b)** weighted.

Note: In a phylogenetic tree built with 16S rRNA sequence data from all samples, the UniFrac distances represent the fraction of branch length that is shared by any two samples' communities. Smaller UniFrac distances indicate a higher fraction of shared branches between samples, *i.e.*, phylogenetically more similar bacterial communities.

Table S 5.1 Relative abundance of rare phyla (abundance < 0.1 %) in soil samples A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}.

	Relative abundance (% of sequences)									
	A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
	mean	SD	mean	SD	Mean	SD	mean	SD	mean	SD
Spirochaetes	0.00	0.00	0.05	0.06	0.20	0.06	0.05	0.06	0.06	0.06
WS3	0.05	0.04	0.03	0.04	0.08	0.07	0.11	0.04	0.06	0.02
GN02	0.03	0.04	0.05	0.02	0.11	0.04	0.03	0.02	0.04	0.00
GOUTA4	0.01	0.02	0.06	0.04	0.00	0.00	0.00	0.00	0.08	0.07
SC4	0.00	0.00	0.00	0.00	0.01	0.02	0.06	0.06	0.06	0.06
WS2	0.00	0.00	0.02	0.04	0.00	0.00	0.01	0.02	0.04	0.04
GAL15	0.01	0.02	0.00	0.00	0.04	0.04	0.01	0.02	0.01	0.02
Tenericutes	0.00	0.00	0.00	0.00	0.03	0.04	0.03	0.04	0.01	0.02
WS4	0.00	0.00	0.03	0.04	0.03	0.04	0.01	0.02	0.00	0.00
WPS-2	0.01	0.02	0.01	0.02	0.01	0.02	0.00	0.00	0.03	0.02
WS6	0.03	0.02	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.00
WYO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04
BRC1	0.00	0.00	0.01	0.02	0.00	0.00	0.01	0.02	0.00	0.00
MVS-104	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.01	0.02
OP3	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.01	0.02
GN04	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00
OC31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02
TM6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02
WS5	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00
ZB3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02

***** Values are means ± standard deviation (n=3)

Table S 5.2 Relative abundance of bacterial classes with abundances higher than 0.1 % in at least one of the soil samples (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}).

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Class	mean	SD	Mean	SD	Mean	SD	mean	SD	mean	SD
Acidobacteria	*Acidobacteria*	16.90	1.43	15.81	0.24	12.39	1.89	16.28	1.96	18.44	2.12
	"*Solibacteres*"	10.90	0.81	11.48	0.66	5.02	0.02	13.68	0.97	13.97	1.38
	"*Chloracidobacteria*"	3.11	0.27	2.67	0.38	1.62	0.07	3.76	0.43	3.33	0.46
	"*Acidobacteria*-2"	1.11	0.29	1.40	0.30	3.66	0.25	0.93	0.10	1.29	0.23
	"TM1"	0.35	0.08	0.20	0.15	0.39	0.12	0.18	0.06	0.19	0.06
	"MVS-40"	0.03	0.02	0.09	0.02	0.09	0.06	0.28	0.02	0.26	0.14
	"iii1-8"	0.08	0.08	0.03	0.02	0.06	0.06	0.15	0.11	0.10	0.02
	"*Acidobacteria*-6"	0.03	0.02	0.04	0.04	0.05	0.02	0.06	0.02	0.11	0.04
	"PAUC37f"	0.00	0.00	0.05	0.02	0.00	0.00	0.10	0.04	0.06	0.02
Actinobacteria	*Actinobacteria*	4.43	0.53	3.55	0.02	4.05	0.82	3.86	0.16	3.36	0.40
	Acidimicrobiia	3.18	0.28	3.25	0.18	3.15	0.52	2.60	0.42	2.26	0.37
	"MB-A2-108"	0.32	0.29	0.29	0.13	1.05	0.13	0.48	0.12	0.20	0.09
	Thermoleophilia	0.27	0.10	0.26	0.07	0.35	0.12	0.29	0.06	0.19	0.04
AD3	"ABS-6"	0.20	0.08	0.20	0.04	1.61	0.34	0.19	0.08	0.13	0.04
	"JG37-AG-4"	0.01	0.02	0.00	0.00	0.16	0.12	0.00	0.00	0.01	0.02

Table S 5.2 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Class	mean	SD	mean	SD	Mean	SD	mean	SD	mean	SD
Armatimonadetes	"*Fimbriimonadetes*"	0.03	0.02	0.06	0.06	0.24	0.22	0.08	0.04	0.18	0.10
	Chthonomonadetes	0.01	0.02	0.03	0.04	0.04	0.07	0.10	0.10	0.01	0.02
Bacteroidetes	*Sphingobacteriia*	8.28	1.84	5.65	0.87	5.88	0.48	4.87	0.97	3.95	0.47
	Bacteroidia	0.18	0.10	1.32	0.26	0.90	0.33	2.27	0.23	1.39	0.25
	Flavobacteriia	0.63	0.25	0.18	0.04	0.15	0.10	0.10	0.04	0.11	0.08
Chlorobi	"SJA-28"	0.27	0.07	0.46	0.19	0.78	0.24	0.66	0.15	0.62	0.26
	"BSV26"	0.00	0.00	0.15	0.16	0.13	0.06	0.43	0.09	0.33	0.13
	"OPB56"	0.01	0.02	0.10	0.04	0.05	0.04	0.16	0.04	0.14	0.06
Chloroflexi	*Anaerolineae*	2.01	0.70	3.30	0.38	2.80	0.85	5.21	0.55	5.87	1.35
	"Ellin6529"	2.33	0.22	2.88	0.45	5.40	0.67	2.27	0.54	2.07	0.29
	"Thermobacula"	0.44	0.02	0.33	0.04	0.53	0.14	0.40	0.15	0.33	0.14
	"Bljii12"	0.27	0.07	0.41	0.26	0.45	0.14	0.39	0.22	0.25	0.06
	"TK17"	0.15	0.11	0.28	0.11	0.39	0.13	0.16	0.13	0.15	0.08
	Ktedonobacteria	0.19	0.10	0.14	0.08	0.41	0.07	0.14	0.04	0.16	0.02
	Chloroflexi	0.13	0.06	0.08	0.07	0.43	0.18	0.15	0.10	0.25	0.06
	Thermomicrobia	0.22	0.08	0.11	0.04	0.11	0.07	0.13	0.06	0.09	0.06
	"S085"	0.14	0.06	0.04	0.04	0.14	0.02	0.08	0.08	0.05	0.04

Table S 5.2 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Class	mean	SD	Mean	SD	Mean	SD	mean	SD	mean	SD
Cyanobacteria	*Nostocophycideae*	0.13	0.08	0.15	0.07	1.10	0.27	0.08	0.04	0.14	0.09
	Oscillatoriophycideae	0.25	0.23	0.05	0.04	0.06	0.04	0.08	0.08	0.06	0.06
	Synechococcophycideae	0.03	0.02	0.14	0.04	0.11	0.10	0.00	0.00	0.08	0.08
	"S15B-MN24"	0.03	0.04	0.02	0.04	0.11	0.04	0.05	0.06	0.06	0.08
Elusimicrobia	*Elusimicrobia*	0.52	0.21	0.70	0.08	1.22	0.43	0.68	0.28	1.20	0.24
	Endomicrobia	0.04	0.00	0.13	0.02	0.04	0.00	0.11	0.07	0.13	0.06
Fibrobacteres	*Fibrobacteria*	0.08	0.04	0.06	0.04	0.31	0.23	0.00	0.00	0.04	0.04
Firmicutes	*Clostridia*	0.43	0.14	1.18	0.21	1.38	0.21	0.64	0.13	0.53	0.17
	Bacilli	0.11	0.04	0.07	0.13	0.15	0.07	0.18	0.09	0.11	0.08
Gemmatimonadetes	*Gemmatimonadetes*	4.78	0.23	5.06	0.68	3.85	0.44	5.52	0.39	4.98	0.45
	"Gemm-1"	1.89	0.41	2.31	0.11	2.01	0.18	1.34	0.53	1.46	0.40
Nitrospira	Nitrospira	2.14	0.37	3.97	0.48	2.85	0.47	1.98	0.31	2.59	0.67
OD1	"SM2F11"	0.10	0.10	0.11	0.07	0.85	0.19	0.09	0.02	0.19	0.10
OP11	"OP11-3"	0.03	0.04	0.05	0.04	0.19	0.10	0.01	0.02	0.08	0.04

Table S 5.2 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Class	mean	SD	mean	SD	Mean	SD	mean	SD	mean	SD
Proteobacteria	*Alphaproteobacteria*	26.49	1.46	24.05	0.12	23.53	1.41	21.12	1.77	21.35	1.20
	Deltaproteobacteria	1.09	0.22	1.33	0.20	1.88	0.55	1.49	0.12	1.35	0.25
	Betaproteobacteria	1.13	0.27	1.13	0.17	0.87	0.08	1.48	0.10	0.96	0.12
	Gammaproteobacteria	0.42	0.20	0.29	0.11	0.16	0.08	0.23	0.00	0.16	0.08
	Epsilonproteobacteria	0.00	0.00	0.10	0.12	0.00	0.00	0.08	0.08	0.20	0.04
Spirochaetes	"*Leptospirae*"	0.00	0.00	0.05	0.06	0.20	0.06	0.04	0.04	0.04	0.04
TM7	"TM7-1"	0.65	0.07	0.23	0.22	1.20	0.14	0.26	0.20	0.18	0.06
	"SC3"	0.11	0.07	0.03	0.04	0.25	0.09	0.09	0.04	0.04	0.04
WS3	"PRR-12"	0.05	0.04	0.03	0.04	0.08	0.07	0.11	0.04	0.06	0.02

* Values are means ± standard deviation (n=3)

Table S 5.3 Relative abundance of bacterial orders with abundances higher than 0.1 % in at least one of the soil samples (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}).

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Order	mean	SD	Mean	SD	mean	SD	mean	SD	mean	SD
Acidobacteria	*Acidobacteriales*	16.90	1.43	15.81	0.24	12.39	1.89	16.28	1.96	18.44	2.12
	"*Solibacterales*"	10.90	0.81	11.48	0.66	5.02	0.02	13.68	0.97	13.97	1.38
Actinobacteria	*Actinomycetales*	4.38	0.59	3.08	0.14	3.91	0.70	3.42	0.20	3.01	0.30
	Acidimicrobiales	3.18	0.28	3.25	0.18	3.15	0.52	2.60	0.42	2.26	0.37
	Coriobacteriales	0.05	0.06	0.46	0.15	0.14	0.11	0.44	0.15	0.35	0.14
	Gaiellales	0.20	0.02	0.24	0.04	0.33	0.12	0.24	0.02	0.16	0.04
Armatimonadetes	*Fimbriimonadales*	0.03	0.02	0.06	0.06	0.24	0.22	0.08	0.04	0.18	0.10
	Chthonomonadales	0.01	0.02	0.03	0.04	0.04	0.07	0.10	0.10	0.01	0.02
Bacteroidetes	*Bacteroidales*	0.18	0.10	1.32	0.26	0.90	0.33	2.27	0.23	1.39	0.25
	Flavobacteriales	0.62	0.24	0.18	0.04	0.15	0.10	0.10	0.04	0.11	0.08
	Sphingobacteriales	8.28	1.84	5.65	0.87	5.88	0.48	4.87	0.97	3.95	0.47
Chloroflexi	"envOPS12"	0.51	0.26	0.65	0.19	0.70	0.30	1.59	0.52	1.66	0.49
	"SBR1031"	0.54	0.21	1.00	0.18	0.93	0.32	0.67	0.18	1.25	0.20
	Anaerolineales	0.20	0.10	0.50	0.34	0.30	0.10	0.91	0.20	0.76	0.33
	"A31"	0.18	0.08	0.38	0.06	0.43	0.28	0.42	0.13	0.49	0.21

Table S 5.3 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Order	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
	"SJA-15"	0.11	0.04	0.18	0.18	0.13	0.06	0.63	0.18	0.72	0.40
	"*Roseiflexales*"	0.13	0.06	0.08	0.07	0.41	0.17	0.15	0.10	0.25	0.06
	"H39"	0.11	0.07	0.18	0.06	0.11	0.07	0.26	0.06	0.30	0.10
	"WCHB1-50"	0.13	0.06	0.10	0.06	0.05	0.02	0.23	0.08	0.23	0.14
	"JG30-KF-CM45"	0.14	0.08	0.06	0.02	0.08	0.04	0.11	0.08	0.08	0.04
	"GCA004"	0.05	0.02	0.04	0.04	0.05	0.09	0.10	0.08	0.18	0.09
	"pLW-97"	0.06	0.08	0.05	0.06	0.03	0.02	0.15	0.10	0.11	0.04
	Thermogemmatisporales	0.05	0.02	0.10	0.04	0.15	0.10	0.03	0.02	0.05	0.04
	"CFB-26"	0.04	0.04	0.11	0.06	0.01	0.02	0.04	0.04	0.09	0.09
Cyanobacteria	*Nostocales*	0.13	0.08	0.14	0.04	1.08	0.29	0.06	0.04	0.13	0.09
	Oscillatoriales	0.25	0.23	0.04	0.04	0.00	0.00	0.03	0.04	0.03	0.02
	Pseudanabaenales	0.03	0.02	0.06	0.08	0.10	0.11	0.00	0.00	0.03	0.04
Elusimicrobia	*"FAC88"*	0.27	0.16	0.35	0.06	0.75	0.20	0.24	0.06	0.43	0.19
	"IIb"	0.14	0.08	0.14	0.11	0.16	0.19	0.18	0.17	0.42	0.14
	Elusimicrobiales	0.11	0.04	0.15	0.06	0.24	0.04	0.20	0.13	0.20	0.13
	"MVP-88"	0.00	0.00	0.03	0.02	0.04	0.04	0.04	0.04	0.11	0.10

Table S 5.3 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Order	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Fibrobacteres	"258ds10"	0.08	0.04	0.06	0.04	0.31	0.23	0.00	0.00	0.04	0.04
Firmicutes	*Clostridiales*	0.35	0.13	1.02	0.23	1.32	0.23	0.57	0.08	0.49	0.13
	"OPB54"	0.08	0.00	0.15	0.04	0.04	0.00	0.06	0.06	0.04	0.04
Gemmatimonadetes	"N1423WL"	1.68	0.19	1.72	0.31	1.25	0.25	1.97	0.30	1.79	0.11
	Gemmatimonadales	1.42	0.17	1.43	0.20	0.68	0.17	1.41	0.30	1.13	0.13
	"Ellin5290"	0.56	0.15	0.63	0.13	0.48	0.24	0.97	0.02	0.78	0.12
	"KD8-87"	0.09	0.02	0.21	0.04	0.06	0.04	0.05	0.06	0.11	0.04
Nitrospira	*Nitrospirales*	2.14	0.37	3.97	0.48	2.85	0.47	1.98	0.31	2.59	0.67
Planctomycetes	"*Gemmatales*"	0.14	0.06	0.04	0.04	0.01	0.02	0.09	0.02	0.01	0.02
Proteobacteria	*Sphingomonadales*	12.61	0.87	13.17	0.76	10.09	0.54	10.38	1.73	10.86	1.49
	Rhizobiales	6.09	0.17	5.08	0.74	5.40	0.30	5.15	0.23	4.64	0.62
	Rhodospirillales	3.68	0.33	3.22	0.28	3.70	0.53	3.41	0.40	3.79	0.09
	Caulobacterales	1.43	0.14	1.00	0.28	1.33	0.22	0.81	0.22	0.71	0.31
	"Ellin329"	1.27	0.07	0.65	0.12	0.90	0.21	0.66	0.27	0.63	0.08
	Myxococcales	0.62	0.21	0.56	0.16	1.24	0.36	0.59	0.25	0.76	0.06
	Rickettsiales	0.46	0.16	0.23	0.06	1.23	0.30	0.18	0.06	0.29	0.02

Table S 5.3 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Order	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
	Rhodobacterales	0.51	0.10	0.38	0.08	0.39	0.08	0.15	0.10	0.18	0.06
	"SC-I-84"	0.33	0.12	0.26	0.15	0.18	0.06	0.37	0.13	0.34	0.08
	Burkholderiales	0.30	0.13	0.18	0.04	0.19	0.11	0.19	0.04	0.06	0.02
	"Ellin6067"	0.23	0.14	0.23	0.07	0.16	0.09	0.18	0.12	0.11	0.10
	Xanthomonadales	0.28	0.12	0.13	0.09	0.10	0.06	0.10	0.02	0.13	0.06
	"BD7-3"	0.13	0.06	0.08	0.00	0.08	0.04	0.14	0.08	0.14	0.04
	Bdellovibrionales	0.13	0.06	0.11	0.13	0.08	0.08	0.06	0.04	0.06	0.04
	Syntrophobacterales	0.11	0.04	0.05	0.04	0.10	0.09	0.10	0.02	0.05	0.02
	Campylobacterales	0.00	0.00	0.10	0.12	0.00	0.00	0.08	0.08	0.20	0.04
	"A21b"	0.04	0.04	0.08	0.04	0.05	0.02	0.14	0.14	0.04	0.04

***** Values are means ± standard deviation (n=3)

Table S 5.4 Relative abundance of bacterial families with abundances higher than 0.1 % in at least one of the soil samples (A_{Apr}/s_{ep}, $ANSs_{ep}$, B_{Apr} and B_{Sep}).

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Family	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Acidobacteria	"*Koribacteraceae*"	16.17	1.64	15.25	0.40	11.97	1.90	15.85	2.01	18.09	2.10
	"*Solibacteraceae*"	10.90	0.81	11.48	0.66	5.02	0.02	13.68	0.97	13.97	1.38
	Acidobacteriaceae	0.71	0.17	0.46	0.10	0.40	0.11	0.34	0.11	0.30	0.07
Actinobacteria	*Intrasporangiaceae*	0.62	0.15	0.46	0.11	0.60	0.13	0.48	0.14	0.48	0.24
	"C111"	0.62	0.18	0.68	0.13	0.33	0.13	0.37	0.12	0.38	0.25
	Micrococcaceae	0.24	0.13	0.26	0.06	0.46	0.12	0.29	0.10	0.20	0.06
	Coriobacteriaceae	0.05	0.06	0.46	0.15	0.14	0.11	0.44	0.15	0.35	0.14
	Frankiaceae	0.27	0.08	0.28	0.08	0.39	0.16	0.28	0.12	0.15	0.08
	Mycobacteriaceae	0.16	0.16	0.28	0.06	0.23	0.16	0.30	0.14	0.25	0.02
	Streptomycetaceae	0.29	0.04	0.25	0.09	0.14	0.04	0.32	0.04	0.21	0.19
	Gaiellaceae	0.18	0.04	0.24	0.04	0.30	0.11	0.21	0.02	0.16	0.04
	Micromonosporaceae	0.30	0.11	0.13	0.08	0.25	0.06	0.19	0.07	0.13	0.12
	Geodermatophilaceae	0.14	0.06	0.15	0.16	0.08	0.08	0.14	0.06	0.09	0.06
	Sporichthyaceae	0.10	0.08	0.06	0.04	0.20	0.09	0.06	0.02	0.06	0.02
	Cellulomonadaceae	0.14	0.06	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00

Table S 5.4 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Family	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Armatimonadetes	*Fimbriimonadaceae*	0.00	0.00	0.00	0.00	0.24	0.22	0.06	0.04	0.16	0.12
Bacteroidetes	*Chitinophagaceae*	5.00	0.85	3.61	0.74	2.86	0.49	3.04	0.56	2.55	0.56
	Flammeovirgaceae	0.59	0.33	0.21	0.06	0.55	0.23	0.28	0.19	0.20	0.08
	Saprospiraceae	0.48	0.19	0.31	0.06	0.13	0.02	0.20	0.15	0.11	0.10
	Flavobacteriaceae	0.62	0.24	0.18	0.04	0.15	0.10	0.10	0.04	0.11	0.08
	Sphingobacteriaceae	0.33	0.08	0.10	0.02	0.24	0.09	0.09	0.09	0.04	0.04
	Flexibacteraceae	0.49	0.29	0.04	0.04	0.08	0.04	0.06	0.04	0.06	0.06
Chloroflexi	*Anaerolinaceae*	0.20	0.10	0.50	0.34	0.30	0.10	0.91	0.20	0.76	0.33
Cyanobacteria	*Nostocaceae*	0.13	0.08	0.14	0.04	1.07	0.30	0.06	0.04	0.13	0.09
Firmicutes	*Clostridiaceae*	0.33	0.17	0.80	0.32	1.10	0.22	0.39	0.09	0.38	0.11
Gemmatimonadetes	"Ellin5301"	1.33	0.11	1.34	0.19	0.66	0.17	1.41	0.30	1.10	0.17
Nitrospira	*Nitrospiraceae*	1.61	0.25	1.70	0.14	2.21	0.41	1.21	0.06	0.90	0.16
	"*Thermodesulfovibrionaceae*"	0.53	0.30	2.23	0.61	0.61	0.14	0.77	0.30	1.66	0.50
Proteobacteria	Sphingomonadaceae	10.87	0.43	11.97	1.09	9.29	0.37	10.03	1.61	10.52	1.45
	Rhodospirillaceae	2.96	0.33	2.63	0.20	3.22	0.60	2.85	0.35	3.23	0.25
	Hyphomicrobiaceae	2.18	0.35	2.33	0.20	2.32	0.06	2.66	0.35	2.50	0.52
	Caulobacteraceae	1.43	0.14	0.99	0.29	1.30	0.18	0.78	0.22	0.69	0.33

Table S 5.4 Continued

		Relative abundance (% of sequences)									
		A_{Apr}		A_{Sep}		ANS_{Sep}		B_{Apr}		B_{Sep}	
Phylum	Family	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
	Bradyrhizobiaceae	0.80	0.14	0.40	0.18	0.92	0.19	0.42	0.11	0.44	0.13
	Phyllobacteriaceae	0.53	0.30	0.45	0.10	0.18	0.08	0.30	0.11	0.16	0.06
	Acetobacteraceae	0.22	0.10	0.14	0.06	0.21	0.04	0.23	0.08	0.25	0.12
	Cystobacteraceae	0.14	0.02	0.11	0.08	0.39	0.12	0.14	0.02	0.23	0.10
	Hyphomonadaceae	0.29	0.09	0.25	0.06	0.23	0.04	0.10	0.11	0.13	0.06
	"Mitochondria"	0.05	0.06	0.11	0.07	0.50	0.25	0.04	0.04	0.10	0.06
	Rhizobiaceae	0.30	0.17	0.08	0.00	0.21	0.09	0.13	0.12	0.00	0.00
	Methylocystaceae	0.15	0.04	0.15	0.16	0.13	0.06	0.16	0.08	0.15	0.00
	Comamonadaceae	0.23	0.20	0.13	0.06	0.15	0.08	0.15	0.04	0.05	0.02
	Erythrobacteraceae	0.34	0.33	0.09	0.02	0.09	0.02	0.13	0.04	0.00	0.00
	Beijerinckiaceae	0.11	0.10	0.16	0.10	0.15	0.08	0.15	0.08	0.11	0.00
	Haliangiaceae	0.15	0.07	0.11	0.04	0.21	0.09	0.11	0.04	0.05	0.04
	Rhodobacteraceae	0.22	0.12	0.13	0.08	0.16	0.08	0.00	0.00	0.05	0.02
	Myxococcaceae	0.00	0.00	0.10	0.06	0.19	0.17	0.13	0.04	0.15	0.10
	Rhodobiaceae	0.10	0.04	0.06	0.02	0.13	0.16	0.14	0.02	0.13	0.02
	Xanthomonadaceae	0.15	0.08	0.11	0.06	0.08	0.07	0.06	0.04	0.10	0.02

***** Values are means ± standard deviation (n=3)

Table S 5.5 Number of representative OTUs varying ($P < 0.05$) among cropped and uncropped soils.

	Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
	OTUs present at least in one sample at abundance > 0.1 %									
Absent in soil ANS_{Sep}	Bacteria	11	*Acidobacteria*	5	"*Solibacteres*"	3	"*Solibacterales*"	3	"*Solibacteraceae*"	3
					"*Chloracidobacteria*"	1				
					"TM1"	1				
			Chlorobi	3	"SJA-28"	3				
			Chloroflexi	1	*Anaerolineae*	1	"SJA-15"	1		
			Proteobacteria	2	*Alphaproteobacteria*	2	*Sphingomonadales*	1	*Sphingomonadaceae*	1
							Rhizobiales	1	*Phyllobacteriaceae*	1
	OTUs present in all the samples at abundance > 0.1 %									
Decreased in soil ANS_{Sep}	Bacteria	11	*Acidobacteria*	7	"*Chloracidobacteria*"	1				
					"*Solibacteres*"	4	"*Solibacterales*"	4	"*Solibacteraceae*"	4
					Acidobacteria	2	*Acidobacteriales*	2	"*Koribacteraceae*"	2
			Proteobacteria	3	*Alphaproteobacteria*	3	*Sphingomonadales*	1	*Sphingomonadaceae*	1
							Rhizobiales	1	*Hyphomicrobiaceae*	1
							Rhodospirillales	1	*Rhodospirillaceae*	1

Table S 5.5 Continued

	Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
					OTUs present at least in one sample at abundance > 0.1 %					
Decreased in soil ANS_{Sep}	Bacteria	11	*Acidobacteria*	5	"*Solibacteres*"	2	"*Solibacterales*"	2	"*Solibacteraceae*"	2
					Acidobacteria	3	*Acidobacteriales*	3	"*Koribacteraceae*"	3
			Chloroflexi	2	*Anaerolineae*	2	"envOPS12"	2		
			Gemmatimonadetes	2	*Gemmatimonadetes*	2	*Gemmatimonadales*	1	"Ellin 5301"	1
							"N1423WL"	1		
			Proteobacteria	2	*Alphaproteobacteria*	2	*Sphingomonadales*	1	*Sphingomonadaceae*	1
							Rhizobiales	1		
					OTUs present at least in one sample at abundance > 0.1 %					
Only in soil ANS_{Sep}	Bacteria	5	*Acidobacteria*	1	*Acidobacteria*	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
				1	"*Acidobacteria* - 2"	1				
			AD3	1						
			Chloroflexi	1	"Ellin 6529"	1				
			OD1	1						

Table S 5.5 Continued

Increased in soil ANS_{Sep}

Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
OTUs present in all the samples at abundance > 0.1 %									
Bacteria	7	*Acidobacteria*	3	"*Acidobacteria* - 2 "	2				
				Acidobacteria	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
		AD3	1	"ABS-6"	1				
		Gemmatimonadetes	1	*Gemmatimonadetes*	1				
		Proteobacteria	2	*Deltaproteobacteria*	1	*Myxococcales*	1	*Cystobacteraceae*	1
				Alphaproteobacteria	1	*Rhizobiales*	1	*Bradyrhizobiaceae*	1
OTUs present at least in one sample at abundance > 0.1 %									
Bacteria	33	*Acidobacteria*	6	"Acidobacteria - 2 "	6				
		Actinobacteria	2	*Actinobacteria*	1	*Actinomycetales*	1	*Kineosporiaceae*	1
				Acidimicrobiia	1	*Acidimicrobiales*	1	"EB1017"	1
		AD3	1	"ABS-6"	1				
		Bacteroidetes	1	*Sphingobacteriia*	1	*Sphingobacteriales*	1		
		Chlorobi	1	""SJA-28""	1				
		Chloroflexi	7	"Ellin 6529"	7				

Table S 5.5 Continued

	Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
Increased in soil ANS_{Sep}			*Cyanobacteria*	2	*Nostocophycideae*	2	*Nostocales*	2	*Nostocaceae*	2
			Fibrobacteres	1	*Fibrobacteria*	1				
			Gemmatimonadetes	3	"Gemm-1"	2				
					Gemmatimonadetes	1				
			Nitrospira	1	*Nitrospira*	1	*Nitrospirales*	1	*Nitrospiraceae*	1
			OD1	1						
			Proteobacteria	5	*Alphaproteobacteria*	5	*Sphingomonadales*	1	*Sphingomonadaceae*	1
							Caulobacterales	1	*Caulobacteraceae*	1
							Rhodospirillales	1	*Rhodospirillaceae*	1
							Rickettsiales	2	"Mitochondria"	1
			TM7	1						

Table S 5.6 Number of representative OTUs varying ($P < 0.05$) with crop rotation and management

	Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
Only in soil A_{Apr}	OTUs present at least in one sample at abundance > 0.1 %									
	Bacteria	5	*Actinobacteria*	1	*Actinobacteria*	1	*Actinomycetales*	1	*Cellulomonadaceae*	1
			Bacteroidetes	4	*Sphingobacteriia*	4	*Sphingobacteriales*	4	*Chitinophagaceae*	2
									Flexibacteraceae	1
									Saprospiraceae	1
Dominant in soil: A_{apr}	OTUs present in all the samples at abundance > 0.1 %									
	Bacteria	1	*Proteobacteria*	1	*Alphaproteobacteria*	1	*Rhizobiales*	1	*Hyphomicrobiaceae*	1
	OTUs present at least in one sample at abundance > 0.1 %									
	Bacteria	7	*Bacteroidetes*	4	*Sphingobacteriia*	3	*Sphingobacteriales*	3	*Chitinophagaceae*	2
									Flexibacteraceae	1
					Flavobacteriia	1	*Flavobacteriales*	1	*Flavobacteriaceae*	1
			Proteobacteria	2	*Alphaproteobacteria*	2	*Sphingomonadales*	1		
							Caulobacterales	1	*Caulobacteraceae*	1
			Acidobacteria	1	"TM1"	1				

Table S 5.6 Continued

		Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
Dominant in soil:	A_{Sep}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	3	*Nitrospira*	3	*Nitrospira*	3	*Nitrospirales*	3	*Nitrospiraceae*	2
										"*Thermodesulfovibrionaceae*"	1
	A_{Apr}/A_{sep}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	2	*Proteobacteria*	1	*Alphaproteobacteria*	1	*Sphingomonadales*	1		
				Bacteroidetes	1	*Sphingobacteriia*	1	*Sphingobacteriales*	1	*Saprospiraceae*	1
Decrease from Soil A_{Apr} to B_{Sep}		OTUs present in all the samples at abundance > 0.1 %									
		Bacteria	2	*Acidobacteria*	1	"*Solibacteres*"	1	"*Solibacterales*"	1	"*Solibacteraceae*"	1
				Proteobacteria	1	*Alphaproteobacteria*	1	*Caulobacterales*	1	*Caulobacteraceae*	1
		OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	10	*Acidobacteria*	4	"*Solibacteres*"	2	"*Solibacterales*"	2	"*Solibacteraceae*"	2
						Acidobacteria	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
						"*Chloracidobacteria*"	1				
				Actinobacteria	1	*Actinobacteria*	1	Actinomycetales	1		
				Proteobacteria	4	*Alphaproteobacteria*	4	*Sphingomonadales*	2	*Sphingomonadaceae*	2
								Caulobacterales	1	*Caulobacteraceae*	1
								Rhizobiales	1		
				Bacteroidetes	1	*Sphingobacteriia*	1	*Sphingobacteriales*	1	*Chitinophagaceae*	1

Table S 5.6 Continued

Increase from Soil A_{Apr} to B_{Sep}

Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
OTUs present in all the samples at abundance > 0.1 %									
Bacteria	3	*Acidobacteria*	2	"*Solibacteres*"	1	"*Solibacterales*"	1	"*Solibacteraceae*"	1
				Acidobacteria	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
		Proteobacteria	1	*Alphaproteobacteria*	1	*Sphingomonadales*	1	*Sphingomonadaceae*	1
OTUs present at least in one sample at abundance > 0.1 %									
Bacteria	22	*Acidobacteria*	10	*Acidobacteria*	4	*Acidobacteriales*	4	"*Koribacteraceae*"	4
				"*Solibacteres*"	6	"*Solibacterales*"	6	"*Solibacteraceae*"	6
		Actinobacteria	1	*Actinobacteria*	1	*Actinomycetales*	1		
		Bacteroidetes	2	*Bacteroidia*	2	*Bacteroidales*	2		
		Chlorobi	2	"SJA-28"	2				
		Chloroflexi	3	*Anaerolineae*	3	"envOPS12"	2		
						"SJA-15"	1		
		Gemmatimonadetes	1	*Gemmatimonadetes*	1				
		Nitrospira	1	*Nitrospira*	1	*Nitrospirales*	1	"*Thermodesulfovibrionaceae*"	1
		Proteobacteria	2	*Alphaproteobacteria*	1	*Rhizobiales*	1	*Hyphomicrobiaceae*	1
				Epsilonproteobacteria	1	*Campylobacterales*	1	*Helicobacteraceae*	1

Table S 5.6 Continued

		Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
Dominant in soil:	B_{Apr}/B_{Sep}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	2	*Acidobacteria*	1	"*Chloracidobacteria*"	1				
				Chloroflexi	1	*Anaerolineae*	1	"envOPS12"	1		
		OTUs present in all the samples at abundance > 0.1 %									
		Bacteria	3	*Acidobacteria*	2	"*Solibacteres*"	1	"*Solibacterales*"	1	"*Solibacteraceae*"	1
						Acidobacteria	1	*Acidobacteriales*	1	"*Koribacteraceae*"	1
				Proteobacteria	1	*Alphaproteobacteria*	1	*Rhodospirillales*	1	*Rhodospirillaceae*	1
	B_{Apr}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	5	*Actinobacteria*	1	*Actinobacteria*	1	Actinomycetales	1	*Nocardioidaceae*	1
				Chloroflexi	1	*Anaerolineae*	1	"H39"	1		
				Acidobacteria	2	"*Solibacteres*"	2	"*Solibacterales*"	2	"*Solibacteraceae*"	2
				Bacteroidetes	1	*Bacteroidia*	1	*Bacteroidales*	1		

Table S 5.7 Number of representative OTUs varying ($P < 0.05$) over rice cycle.

Dominant in soil:		Domain	No. OTUs	Phylum	No. OTUs	Class	No. OTUs	Order	No. OTUs	Family	No. OTUs
	A/B_{sep}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	4	*Nitrospira*	3	*Nitrospira*	3	*Nitrospirales*	3	"*Thermodesulfovibrionaceae*"	3
				Gemmatimonadetes	1	*Gemmatimonadetes*	1				
	$A/ANS/B_{Sep}$	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	1	*Nitrospira*	1	*Nitrospira*	1	*Nitrospirales*	1	"*Thermodesulfovibrionaceae*"	1
	A/B_{Apr}	OTUs present at least in one sample at abundance > 0.1 %									
		Bacteria	2	*Gemmatimonadetes*	1	*Gemmatimonadetes*	1	*Gemmatimonadales*	1	"Ellin 5301"	1

Chapter 6

A polyphasic assessment of the effect of an alfalfa-rice rotation system and crop cycle on the paddy soil bacterial community structure and function

Results included in:

Lopes, A.R., D, Prieto-Fernández, A., Trasar-Cepeda, C., Gil-Sotres, F., Leirós, M.C., Manaia, C.M. and Nunes, O.C. A polyphasic assessment of the effect of an alfalfa-rice rotation system and crop cycle on the paddy soil bacterial community structure and function. (submitted for publication)

6.1 Abstract

The bulk soil of two adjacent paddy fields at different stages of an alfalfa-rice crop rotation system was characterized over the annual rice cycle (before seeding, maximum tillering and after harvest) using a polyphasic approach. This study aimed to assess the variations associated with crop rotation and/or rice cycle stages regarding soil microbial activity and bacterial community structure and composition and their relationship with different biotic and abiotic environmental parameters.

The most notorious variations over the rice cycle were the organic matter degradation and N_2 fixation, which were independent of crop rotation stage and were intensified, respectively, before and after flooding. Both transformations contributed to increase the content of NH_4^+-N in paddy soil. The N_2 fixation was positively correlated with the abundance of cultivable diazotrophs, and with OTUs affiliated to presumable diazotrophs (*Chloroflexi*-Ellin6529, *Actinobacteria-Acidomicrobiales/Actinomycetales*, *Betaproteobacteria* and *Alphaproteobacteria-Rhizobiales/Rhodospirilales*). Both the abundance of heterotrophic populations and enzymatic activity contributed to the organic matter degradation.

The stage of crop rotation imposed strong variations on the bacterial community structure and composition, mainly before rice seeding. Low content of water, total C and available inorganic-P and the presence of alfalfa debris favoured the proliferation of the aerobic heterotrophic population and the degradation of complex carbon sources. The lineages *Flavobacteriales*, *Sphingobacteriales*, *Caulobacterales*, *Rhizobiales*, and *Actinomycetales* had probably a major role in these activities. In opposition, high content of water, total C and available inorganic-P and the presence of rice debris and/or organic amendments favoured the proliferation of presumable anaerobic members (*Bacteroidales*, *Chlorobi*, and *Anaerolinea*) and the proteolytic activity.

Keywords: organic farming**,** microbial ecology, CLPP, cultivable populations, DGGE, 454-pyrosequencing, multivariate analyses.

6.2 Introduction

Soil bacterial communities are known to be influenced by a wide range of biotic and abiotic factors. Given the biological, chemical and geological complexity of soils, it is very difficult to know in each case which are the most critical parameters, and in which combination they shape the composition, structure and function of the microbial communities. Soil characteristics such as the parent material, texture, and pH have been considered those most influencing microbial communities (Lauber et al. 2009; Ulrich and Becker 2006; van Diepeningen et al. 2006). Land use history (Stark et al. 2008) and the type of aboveground vegetation (Berg and Smalla 2009; Kowalchuk et al. 2002) are also critical parameters defining the soil bacterial community composition, in particular, in the rhizosphere. However, some authors have demonstrated that the type of aboveground vegetation may induce only minor changes in the soil bacterial community composition (Jangid et al. 2011; Kielak et al. 2008a). For these reasons, a reliable prediction of how microbial communities will react, for instance, which bacterial groups will be outcompeted or favoured, when the soil environment is altered, is really challenging.

Agriculture is the oldest and still important anthropogenic activity. In the last decades, agricultural productivity has increased, mainly due to the use of synthetic chemical compounds such as pesticides and inorganic fertilizers. These compounds have negative impacts on the agriculture ecosystems, the surrounding environment and the human food-chain (Galloway et al. 2008; Hussain et al. 2009; Matson et al. 1997; Quayle et al. 2006; Ramirez et al. 2012). Therefore, nowadays, there is an increasing interest in improving and implementing sustainable practices, such as the ancient crop rotation system. In this farming management at least two different types of crops are sequentially cultivated in the same area (Kelner et al. 1997; Ladha and Reddy 2003; Larkin and Honeycutt 2006; Sun et al. 2009; Yin et al. 2010; Zhao et al. 2009). Without the use of synthetic compounds, this practice contributes to increase the agricultural productivity and soil fertility (Kundu and

Ladha 1995; Liebman and Davis 2000; Rui and Zhang 2010). Among the rotation systems well accepted and most used are those that include a legume in rotation with other crop (e.g. cereal, tuberous). In these systems, the efficiency of the legume-crop rotation will depend on the interaction established between the legume plant and the microbial community, and on the way both benefit and influence the physicochemical and biochemical properties of surrounding environment (Ladha and Reddy 2003; O'Hara et al. 1989; Pietsch et al. 2007).

Rice is amongst the most cultivated crops worldwide (FAOSTAT 2010). Since this cereal is cultivated mainly in flooded lands, the microbiota of rice fields is subjected to unique environmental conditions. Indeed, rice paddies are characterized by an impressive compositional and functional microbial diversity (Ikenaga et al. 2003; Ishii et al. 2009; Noll et al. 2005; Treude et al. 2003), enhanced by the redox gradients created in the soil under the flooding conditions (Schmidt et al. 2011). Recently, it was reported that higher rice productivity could be obtained under crop rotation than under a continuous monoculture of rice (Chen et al. 2012; Xuan et al. 2012). Crop rotation also improves paddy soil quality (Chen et al. 2012) and biological nitrogen fixation, particularly in rice-legume rotation systems (Kundu and Ladha 1995; Ladha and Reddy 2003). It is described that rice crop rotation influences the soil microbial community structure and composition (Chen et al. 2012; Xuan et al. 2012). However, an improved insight of the influence of type of crops in rotation may be obtained from an integrated approach in which the microbial community composition and function are assessed simultaneously. Such studies would bring additional information about the short and long term influence of the crop in rotation on the microbial community composition and functions in the rice paddy soils. This type of knowledge is important not only to get science-based evidences of the advantages of the crop rotation systems, but also to improve these practices.

The use of polyphasic approaches in which the composition of the microbial communities, the functional activity and the physicochemical and biochemical

properties of bulk soil at distinct stages of the organic rice-alfalfa rotation system are characterized simultaneously bring an integrated view of the system. If the same methodology is applied to different stages of the rotation cycle and distinct phases of the rice cycle (before seeding, maximum tillering and after harvesting), then it will be possible to have a general perspective of the transformations taking place. In a previous study, the bacterial communities of rice paddies in different stages of crop rotation were characterized before rice seeding and after harvest using 454-pyrosequencing (Lopes et al., submitted). The current study was designed to get additional inferences about the correlation between microbial community composition and function and different biotic and abiotic environmental parameters, over the rice cycle or crop rotation stage. Thus, to achieve this purpose multivariate analyses were used to assess the i) predominant microbial activities occurring at different stages of alfalfa-rice rotation system and/or over the rice cycle; ii) bacterial community members correlated with the most intense microbial activities; and iii) biotic and abiotic soil parameters most correlated with the microbial populations and soil biological activity variations.

6.3 Material and Methods

6.3.1 Site description and soil sampling

Bulk soil samples were collected from two adjacent paddy fields (A and B) located in the experimental farm "Bico da Barca" (Montemor-o-Velho, central Portugal). In these paddies, rice is cropped in an alfalfa-rice rotation system as described elsewhere (Lopes et al., submitted). Briefly, alfalfa is cropped for two consecutive years (1st and 2nd year of crop rotation) up to the soil preparation for rice cropping, which occurs in April. Rice is cropped in the two following years (3rd and 4th year of crop rotation). The annual rice cycle starts in May, when rice is seeded in the flooded paddies, and ends in late September, when it is harvested. Between the two years of rice cropping, paddies lay fallow in winter. At the sampling time, in 2010,

paddy A and B were, respectively, at the 3rd and 4th year of the crop rotation cycle. Because of the lower rice yield of crops at the 2nd year of rice cropping, in paddy B soil preparation included organic- and inorganic P amendments.

Triplicate composite samples were collected to allow for within-plot variability as previously described (Lopes et al., submitted), at the beginning of April (before seeding, A_{Apr} and B_{Apr}), in the middle of July (maximum tillering, A_{Jul} and B_{Jul}) and in late September (after harvesting, A_{Sep} and B_{Sep}). The detailed procedures for processing and storage of the samples were previously described by Lopes et al. (2011).

6.3.2 Soil physical, chemical and biochemical properties

The total C and N contents, pH in water and water content were determined following the methods described by Guitián and Carballas (1976).Total inorganic-N and NH_4^+-N contents were determined by steam distillation after extraction with 2 M KCl (Bremner 1965). Total and inorganic available-P were determined after extraction with 0.5 M sodium bicarbonate following the methods described in Trasar-Cepeda et al. (1990).

Microbial biomass C (Biomass C) was determined by the chloroform fumigation extraction method, with 0.5 M K_2SO_4 as extractant (Vance et al., 1987). The difference in C content of the fumigated and unfumigated extracts was converted to microbial biomass C by applying a factor *(Kc)* of 0.45 (Jenkinson 1988). Soil basal respiration (microbial respiration) was determined by static incubation (Guitián and Carballas 1976). The CO_2 produced during a 10-day period by 25 g soil samples incubated at field moisture content and 25 ºC was collected in 10 ml of a 1 M NaOH solution, which was then titrated against HCl. The microbial coefficient (qCO_2) was calculated as the ratio between basal respiration and microbial biomass C (Anderson and Domsch 1985). Total inorganic-N and NH_4^+-N produced by mineralization of organic N were estimated by the difference, respectively,

between the total inorganic-N and the NH_4^+-N contents at the end and at the beginning of the 10-day incubation period, as described above.

Dehydrogenase activity was determined as described by Camina et al. (1998). The activities of urease and protease hydrolysing benzoylargininamide (BAA-protease) were determined as described by Nannipieri et al. (1980) . The activity of protease hydrolysing casein (casein-protease) was determined using the modified method of Ladd and Butler (1972), described by Nannipieri et al. (1979).

All determinations were performed in triplicate and the average values were expressed on an oven-dried (105 ºC) soil basis.

6.3.3 Enumeration of total cells and of cultivable microbial populations

The enumeration of total cells from bulk soil was performed by the 4,6-diamidino-2-phenylindole (DAPI) staining method as described by Brunk et al. (1979). For each sample, 10 g of soil were suspended in 90 ml of sterile sodium hexametaphosphate and sodium pyrophosphate solution (1 % and 0.18 %, respectively). The mixture was stirred for 30 min at 200 rpm and was allowed to sediment for 15 min. The suspension was diluted in 10 fold series and 100 µl aliquots were used for the enumeration of total cells as described by Manuel et al. (2007).

The densities of cultivable microorganisms belonging to different physiological groups (listed in Table 6.1) were determined in bulk soil by the most probable number (MPN) technique, using a modified version of the protocol described by Kidd et al. (2008). Briefly, 20 µl aliquots of soil suspensions prepared as described above were used to inoculate microtiter plates containing a selective liquid medium (180 µl per well) and were sequentially diluted in 10-fold series in the same medium. The redox indicator resazurin was used (1 mg l^{-1}) for cultures incubated

under anaerobic conditions. After the incubation periods (Table 6.1), microbial growth was measured (λ = 660 nm) using a microtiter reading system (SpectraMax M2E, Molecular Devices). The presence of nitrate and/or nitrite in the denitrifiers selective medium was detected using the method of Smibert and Krieg (1981). MPN were estimated from the appropriate tables taking into account the volume inoculated and the initial dilution used. All the determinations were performed in triplicate and the average MPN of each soil microbial population was expressed as log MPN g^{-1}dry soil.

Table 6.1 List of selective liquid media and incubation conditions used in this study to enumerate MPN of different cultivable microbial populations.

Microbial populations	Incubation time (week)	Temperature (ºC)	Selective media (references)
Aerobic heterotrophs	1	25	Kidd et al., 2008
Anaerobic heterotrophs	2	28	Kidd et al., 2008
Aerobic ammonifiers	3	25	Kidd et al., 2008
Anaerobic ammonifiers	3	28	Kidd et al., 2008
Denitrifiers	3	30	Nogales et al., 2002
Aerobic diazotrophs	3	30	Cote and Gherna, 1994
Anaerobic diazotrophs*	3	28	Lin et al., 2008

* The medium was supplemented with Na_2CO_3 (20 mg l^{-1})

6.3.4 Catabolic profiling

The catabolic profiling (CLPP) of each sample was assessed using a modification of the method described by Kennedy (1994). Microplates containing 28 different organic carbon substrates (α-cyclodextrin, glycogen, Tween 80, Tween 60, α-$_D$-glucose-1-phosphate, β-phenyl ethylamine, putrescine, $_D$-(+) cellobiose, *N*-acetyl-$_D$-glucosamine, α-lactose, $_D$-(+) xylose, β-methyl $_D$-glucoside, maltose, i-erythritol, glyceraldehyde, $_L$-phenylalanine, $_L$-glutamic acid, $_L$-threonine, $_L$-asparagine, $_L$-

arginine, $_{L}$-serine, galacturonic acid, $_{D}$-galactonic acid δ-lactone, malonic acid, malic acid, α-keto butyric acid, o-hydroxybenzoic acid, p-hydroxybenzoic acid) and prepared as described by Kidd et al. (2008) were inoculated with soil suspensions prepared and sequentially diluted as described above. Substrate utilization was indicated by colour development of the tetrazolium violet redox dye (2,5-diphenyl-3-(α-naphthyl)tetrazolium chloride, 0.15 mM) after 7 d of aerobic incubation at 25 ºC. The total number of C sources utilized was recorded and the number (MPN) of cells grown at each substrate was estimated using tables for 3 replicates per dilution level.

6.3.5 Molecular characterization

Total genomic DNA extraction, assessment of quality and quantity and the PCR amplification of the 16S rRNA gene and DGGE analyses were performed as previously described (Lopes et al. 2011). The comparison of DGGE profiles was performed using the Bionumerics software (version 6.1, Applied Maths). Pattern normalization, assignment of bands to classes and the construction of band-matching tables were done as previously described (Lopes et al. 2011). The band-matching table (band position vs. band intensity), comprising the triplicate DGGE profiles from both paddies, at different sampling periods (April, July and September), was used in further statistical analyses.

The triplicate rarefied 16S rRNA gene 454-pyrosequencing data from samples of soil A and B from April and September (accession number SAMN01908502 to SAMN01908507 and SAMN01908511 to SAMN01908516, respectively) generated previously (Lopes et al., submitted) was used in the present study. This dataset comprised all the OTUs (1790) that were present in at least two of the three replicas of at least one of the samples examined, weighted according to their abundance (number of sequences).

6.3.6 Statistical analyses

Data of physicochemical and biochemical properties, total number of cells and MPN of cultivable microbial populations occurring, over the rice cycle, in each paddy were compared using the two way analysis of variance (ANOVA) and the post-hoc Tukey test was applied when adequate. Comparisons between paddies at each sampling period were performed using the two-sample statistical test (*t*-test). The statistical analyses were done using Excel software package (Microsoft Excel, 2007).

Principal component analyses (PCA) were used to assess the variations in the physicochemical and biochemical paddy soil properties, microbial community composition based on culture dependent (MPN data) and culture independent data (DGGE and pyrosequencing data) and on the microbial catabolic activity (CLPP data) of both paddies, over the rice cycle. Canonical correspondence analyses (CCA) were carried out to elucidate which parameters were most correlated with the variations of the microbial community activity, based on CLPP data (log MPN g^{-1}dry soil), and composition, based on MPN (log MPN g^{-1}dry soil), DGGE (band intensity) and pyrosequencing data (OTU's abundance). The significance of the established relationships between the community data (principal matrix) and the environmental data (second matrix) was tested by Monte Carlo permutations test (n=499). The multivariate analyses (PCA and CCA) were performed with CANOCO (version. 4.5, Microcomputer Power, Ithaca, NY, USA).

6.4 Results

6.4.1 Variations in the physical, chemical and biochemical characteristics of the paddy soils

Over the rice cycle, physical, chemical and biochemical properties varied significantly and differed between both paddies (Table 6.2). The parameters that

most contributed to explain the variation found among samples were identified based on a principal component analysis (PCA) (Fig. 6.1a). In both paddies, April samples were those with the highest values of microbial coefficient (qCO_2) and NH_4^+-N mineralized, and the lowest values of microbial biomass-C and NH_4^+-N content, explaining the relevance of these parameters to distinguish A_{Apr} and B_{Apr} from the other samples, mainly B_{Jul} and B_{Sep}. Other factors, such as the higher proteolytic (casein- and BAA-protease) and urease activity observed in April, mainly in paddy B, than in the other periods in both paddies contributed also to this differentiation. On the other hand, paddies A and B could also be distinguished, mainly along axis 2. Comparatively, samples in the 4th year of the crop rotation (B) presented higher values of the parameters available-P (total and inorganic), water, total N and C soil contents than samples in the 3rd year (A).

Table 6.2 Physicochemical, biochemical and microbiological soil properties of paddies A and B. Values are means ± standard deviation (n=3).

Parameter	A_{Apr}	B_{Apr}	A_{Jul}	B_{Jul}	A_{Sep}	B_{Sep}
			Physicochemical			
pH in water	** 5.97 ± 0.03 ^{B}	** 5.94 ± 0.06 ^{B}	6.27 ± 0.02 ^{C*}	6.03 ± 0.04 ^{B*}	** 5.88 ± 0.02 ^{A*}	** 5.70 ± 0.02 ^{A*}
Water content (%) (g H_2O 100 g^{-1} wet soil)	** 22.4 ± 0.1 ^{A,B*}	** 27.1 ± 0.8 ^{B*}	23.6 ± 0.9 ^{B*}	28.6 ± 1.2 ^{B*}	** 21.8 ± 0.4 ^{A*}	** 23.4 ± 0.2 ^{A*}
Total C (%)	** 1.33 ± 0.10 ^{A*}	** 1.68 ± 0.04 ^{A,B*}	1.39 ± 0.07 ^{A*}	1.75 ± 0.04 ^{B*}	** 1.33 ± 0.13 ^{A*}	** 1.60 ± 0.05 ^{A*}
Total N (%)	** 0.14 ± 0.00 ^{A*}	** 0.15 ± 0.01 ^{A*}	0.14 ± 0.01 ^{A}	0.15 ± 0.00 ^{A}	** 0.14 ± 0.00 ^{A*}	** 0.18 ± 0.00 ^{B*}
Total available-P (mg P kg^{-1})	** 50.7 ± 0.4 ^{B*}	** 80.3 ± 2.4 ^{B*}	36.3 ± 0.7 ^{A*}	55.6 ± 0.2 ^{A*}	** 35.7 ± 0.6 ^{A*}	** 56.4 ± 0.6 ^{A*}
Available inorganic-P (mg P kg^{-1})	35.7 ± 1.9 ^{B*}	65.2 ± 2.2 ^{B*}	31.2 ± 1.0 ^{A*}	51.1 ± 0.8 ^{A*}	28.7 ± 0.9 ^{A*}	52.4 ± 0.4 ^{A*}
Total inorganic-N (mg N kg^{-1})	9.10 ± 0.70 ^{B}	9.57 ± 0.40 ^{C}	4.55 ± 0.70 ^{A}	5.13 ± 0.40 ^{A}	4.90 ± 0.70 ^{A*}	7.70 ± 0.70 ^{B*}
NH_4^+-N (mg NH_4^+-N kg^{-1})	1.52 ± 0.40 ^{A}	1.28 ± 0.40 ^{A}	3.73 ± 0.40 ^{B*}	5.13 ± 0.40 ^{B*}	4.43 ± 1.07 ^{B}	5.13 ± 0.41 ^{B}
			Biochemical			
Dehydrogenase (µmol INTF g^{-1} h^{-1})	0.10 ± 0.00	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.01	0.11 ± 0.02	0.10 ± 0.01
Microbial biomass - C (mg C kg^{-1})	111 ± 13 ^{A}	112 ± 2 ^{A}	138 ± 6 ^{A*}	187 ± 4 ^{B*}	117 ± 43 ^{A}	177 ± 35 ^{B}
qCO_2 (µg CO_2-C released mg^{-1} biomass carbon h^{-1})	4 ± 0 ^{B}	4 ± 2 ^{A}	3 ± 0 ^{A}	3 ± 0 ^{A}	4 ± 1 ^{B}	3 ± 0 ^{A}
Total inorganic-N min (mg N kg^{-1}10 d^{-1})$^{\#}$	0.04 ± 0.65 ^{A}	0.03 ± 0.12 ^{A}	0.49 ± 0.24 ^{A}	0.55 ± 0.69 ^{A}	2.01 ± 0.57 ^{B}	1.03 ± 0.53 ^{A}
NH_4^+-N min (mg NH_4+-N kg^{-1} 10 d^{-1})$^{\#}$	1.09 ± 0.38 ^{A}	1.45 ± 0.37 ^{C}	0.70 ± 0.48 ^{A*}	-0.91 ± 0.69 ^{B*}	0.22 ± 0.74 ^{A*}	-2.51 ± 0.12 ^{A*}
Urease (µmol NH_3 g^{-1} h^{-1})	3.05 ± 0.27 ^{B}	3.23 ± 0.35 ^{C}	1.68 ± 0.25 ^{A*}	2.30 ± 0.08 ^{B*}	1.18 ± 0.14 ^{A*}	1.69 ± 0.04 ^{A*}
Casein-protease (µmol tyrosine $g^{-1}h^{-1}$)	0.58 ± 0.02 ^{B}	0.55 ± 0.02 ^{B}	0.33 ± 0.05 ^{A}	0.23 ± 0.03 ^{A}	0.28 ± 0.06 ^{A}	0.23 ± 0.03 ^{A}
BAA-protease (µmol NH_3 g^{-1} h^{-1})	5.98 ± 0.56 ^{C*}	10.56 ± 1.53 ^{B*}	3.89 ± 0.25 ^{B*}	5.98 ± 0.45 ^{A*}	2.71 ± 0.11 ^{A*}	5.29 ± 0.07 ^{A*}

Table 6.2 Continued

Parameter	A_{Apr}	B_{Apr}	A_{Jul}	B_{Jul}	A_{Sep}	B_{Sep}
			Microbiological			
Total cells (log Cells g^{-1}dry soil)	11.8 ± 0.1 ^{A}	11.4 ± 0.4 ^{A}	11.9 ± 0.1 ^{A}	11.8 ± 0.1 ^{A}	11.8 ± 0.0 ^{A}	11.8 ± 0.0 ^{A}
Aerobic heterotrophs (log MPN g^{-1}dry soil)	7.3 ± 0.2 ^{B*}	6.5 ± 0.1 ^{B*}	6.4 ± 0.2 ^{A*}	6.0 ± 0.1^{A*}	6.6 ± 0.3 ^{A}	6.5 ± 0.1 ^{B}
Aerobic ammonifiers (log MPN g^{-1}dry soil)	6.6 ± 0.2 ^{B}	6.3 ± 0.1 ^{A}	6.2 ± 0.1^{A,B}	5.8 ± 0.3 ^{A}	6.0 ± 0.2 ^{A}	6.0 ± 0.1 ^{A}
Aerobic diazotrophs (log MPN g^{-1}dry soil)	1.8 ± 0.5 ^{A}	2.4 ± 0.2 ^{A}	4.5 ± 0.5 ^{B}	4.2 ± 0.1 ^{B}	5.8 ± 0.5 ^{C}	5.5 ± 0.2 ^{C}
Anaerobic heterotrophs (log MPN g^{-1}dry soil)	5.2 ± 0.1 ^{A}	5.3 ± 0.1 ^{A,B}	5.2 ± 0.2 ^{A}	5.2 ± 0.1 ^{A}	6.0 ± 0.3 ^{B}	5.6 ± 0.3 ^{B}
Anaerobic ammonifiers (log MPN g^{-1}dry soil)	5.8 ± 0.3 ^{A}	5.5 ± 0.2 ^{A}	5.7 ± 0.8 ^{A}	5.3 ± 0.4 ^{A}	6.2 ± 0.2 ^{A*}	5.8 ± 0.2 ^{A*}
Anaerobic diazotrophs (log MPN g^{-1}dry soil)	2.4 ± 0.2 ^{A}	2.9 ± 0.2 ^{A}	3.9 ± 0.1 ^{B*}	3.5 ± 0.1 ^{B*}	4.1 ± 0.1 ^{B*}	3.7 ± 0.1 ^{B*}
Denitrifiers (log MPN g^{-1}dry soil)	5.4 ± 0.6 ^{A}	5.2 ± 0.1 ^{A}	5.8 ± 0.6 ^{A}	5.9 ± 0.1 ^{B}	5.5 ± 0.3 ^{A}	5.6 ± 0.2 ^{A,B}

A-C, Homogeneous subsets within each paddy (A or B), as determined by the Tukey test at $P<0.05$; *, Significant differences between paddies A and B on basis of the two-sample t-test at $P<0.05$; **, Values from Lopes et al., submitted; $^{\#}$, Positive values indicate transformation of organic N forms into inorganic forms (total inorganic-N or NH_4^+-N) and negative values indicate total inorganic-N or NH_4^+-N immobilization.

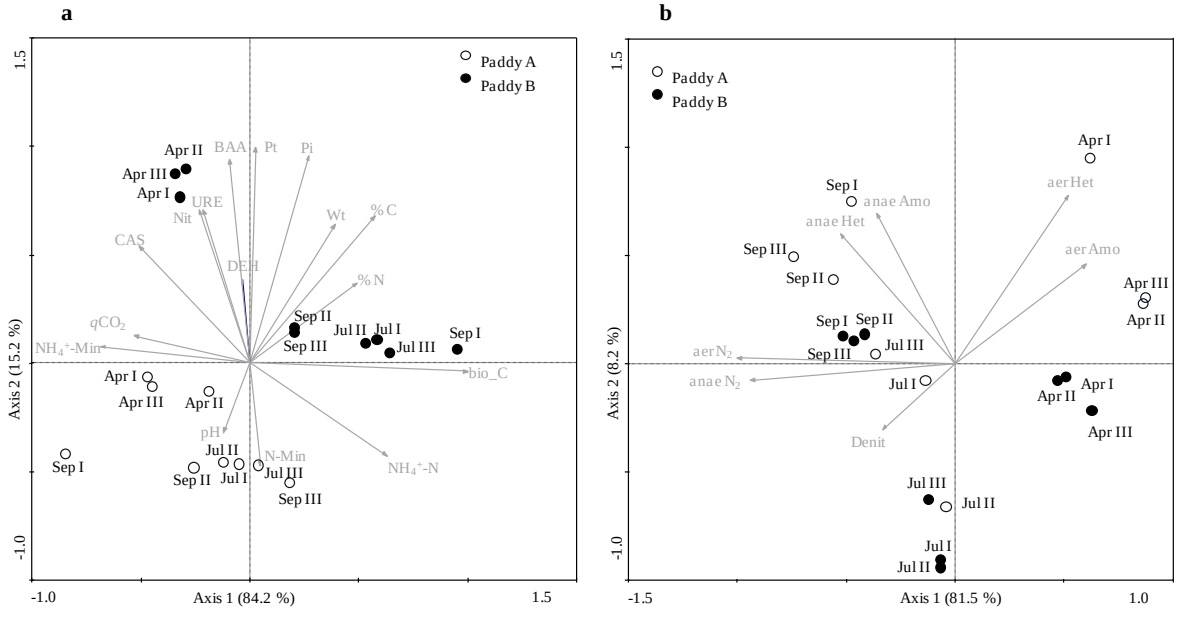

Fig. 6.1 a) PCA biplot of distribution of physicochemical and biochemical soil properties in paddy A and B, over the rice cycle. **b**) PCA biplot of distribution of abundance of different microbial populations in paddy A and B, over the rice cycle.

pH (pH in water); Wt, water content (%); % C, Total C (%); % N, Total N (%); Nit, total inorganic-N (mg N kg^{-1}); NH_4^+, NH_4^+-N (mg NH_4^+-N kg^{-1}); Pt, total available-P (mg P kg^{-1}); Pi, available inorganic-P (mg P kg^{-1}); DEH, dehydrogenase activity (µmol INTF g^{-1} h^{-1}); qCO_2, microbial coefficient (µg CO_2-C released mg^{-1} biomass carbon h^{-1}); URE, urease activity (µmol NH_3 g^{-1} h^{-1}); BAA, BAA-protease activity (µmol NH_3 g^{-1} h^{-1}); CAS, casein-protease activity (µmol tyrosine $g^{-1}h^{-1}$); NH_4-Min, NH_4^+-N mineralized (mg NH_4^+-N $kg^{-1}10d^{-1}$); N-Min, total inorganic-N mineralized (mg inorganic-N $kg^{-1}10d^{-1}$); aer Het, aerobic heterotrophs (log MPN g^{-1}dry soil); anae Het, anaerobic heterotrophs (log MPN g^{-1}dry soil); aer N_2, aerobic diazotrophs (log MPN g-^{1}dry soil); anae N_2, anaerobic diazotrophs (log MPN g^{-1}dry soil); aer Amo, aerobic ammonifiers (log MPN g^{-1}dry soil); anae Amo, anaerobic ammonifiers (log MPN g^{-1}dry soil) and Denit, Denitrifiers (log MPN g^{-1}dry soil).

6.4.2 Variations in the microbial cultivable populations from paddy soils

The cultivable populations constituted a small fraction of the total cells (< 0.002 %). Based on a PCA it was possible to conclude that the composition of the cultivable microbial community was most distinct in April, in both paddies (Fig. 6.1b and Table 6.2). Compared with the other samples, in April both paddies presented a higher abundance of aerobic heterotrophs and aerobic ammonifiers and less aerobic and anaerobic diazotrophs, explaining the variations found (Fig. 6.1b). On the other hand, compared with samples taken in April, the abundance of denitrifiers was higher in July, while that of anaerobic heterotrophs and ammonifiers was higher in September. These differences contributed to distinguish the cultivable populations according to the period of the annual rice cycle.

6.4.3 Physicochemical and biochemical parameters correlated with the variations in the cultivable microbial populations

Since the cultivable microbial populations varied over time, a CCA was performed with the objective of identifying the parameters that might be correlated with those variations (Fig. 6.2). The parameters analysed could explain 87.1 % of the variation of the cultivable populations, 83.3 % of which along axis 1. The abundance of aerobic heterotrophs and ammonifiers was strongly correlated with the activity of casein-protease and urease (0.921 and 0.895 inter-set correlations with axis 1, respectively) (Fig. 6.2a), and contributed to the differentiation of the April samples (Fig. 6.2b). In contrast, the abundance of aerobic and anaerobic diazotrophs, high in July and September, was negatively correlated with the activity of these enzymes.

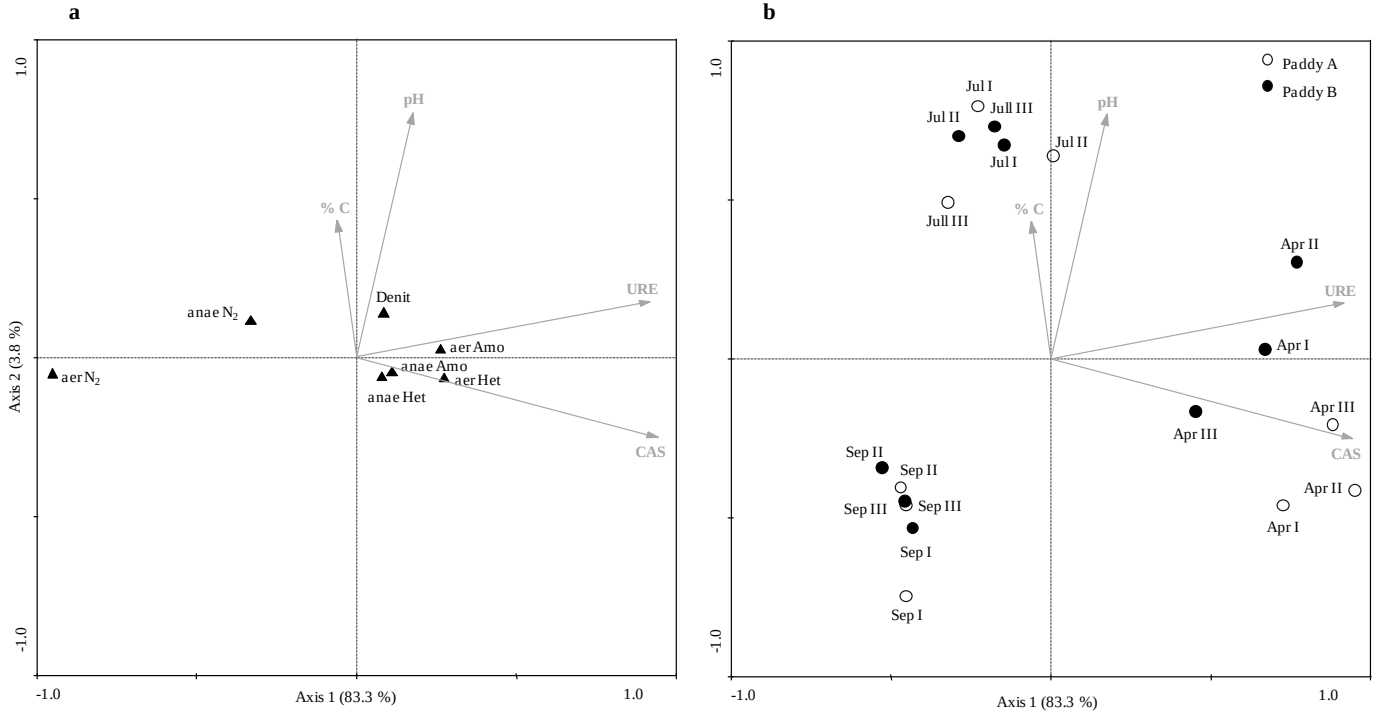

Fig. 6.2 CCA biplot of the variation in the cultivable microbial population (MPN data) in paddy A and B (total inertia 0.018), over the rice cycle, with arrows indicating the corresponding explanatory variables (urease and casein-protease activity, pH and total C content). The species-environmental correlation was 0.975 and 0.896 for axis 1 and 2 respectively. **a**) Representation of the microbial populations distribution. **b**) Representation of samples distribution.

6.4.4 Catabolic profiling of cultivable microbial populations and parameters correlated with its variation

The CLPP analysis aimed to find out phenotypic variations of the microbial communities useful for a temporal and inter-paddy comparison. All the substrates tested supported the aerobic microbial growth in all the samples (Fig. 6.3). In spite of this, it was possible to observe some variations. The abundance of microorganisms growing aerobically at expenses of each substrate was slightly higher in A_{Apr}, which was reflected in the separation of these samples in the corresponding PCA biplot (Fig. 6.4). In contrast, the lowest abundance of microorganisms growing aerobically on most of the substrates occurred in the samples A_{Jul} and B_{Jul}, particularly in sample B_{Jul}.

The biotic and abiotic parameters correlated with the variation of the CLPP profiles were identified using a CCA (Fig. 6.5). The parameters tested could explain 39.1 % of the variance found among the samples, 27.7 % of which over axis 1. The abundance of aerobic heterotrophs and soil water content (-0.766 and 0.838 inter-set correlations with axis 1, respectively) were the parameters that contributed most to the separation of A_{Apr} from $B_{Apr,}$, A_{Jul} and B_{Jul} samples. The abundance of aerobic heterotrophs correlated positively with the abundance of microorganisms growing aerobically on organic acids and polymers, such as o-hydroxybenzoic acid, tween 60, α-cyclodextrin, and glycogen, predominant in samples collected in September (A_{Sep} and B_{Sep}) and mainly in A_{Apr}. On the other hand, the comparatively higher values of water content in samples collected in B_{Apr}, A_{Jul} and mainly B_{Jul}, was negatively correlated with the abundance of aerobic heterotrophs (Table 6.2). According to the CCA, the most abundant aerobic microorganisms, gaining advantage in the periods of highest water content, used amines, aminoacids and carbohydrates, such as putrescine, L-asparagine, and cellobiose, in particular in paddy B.

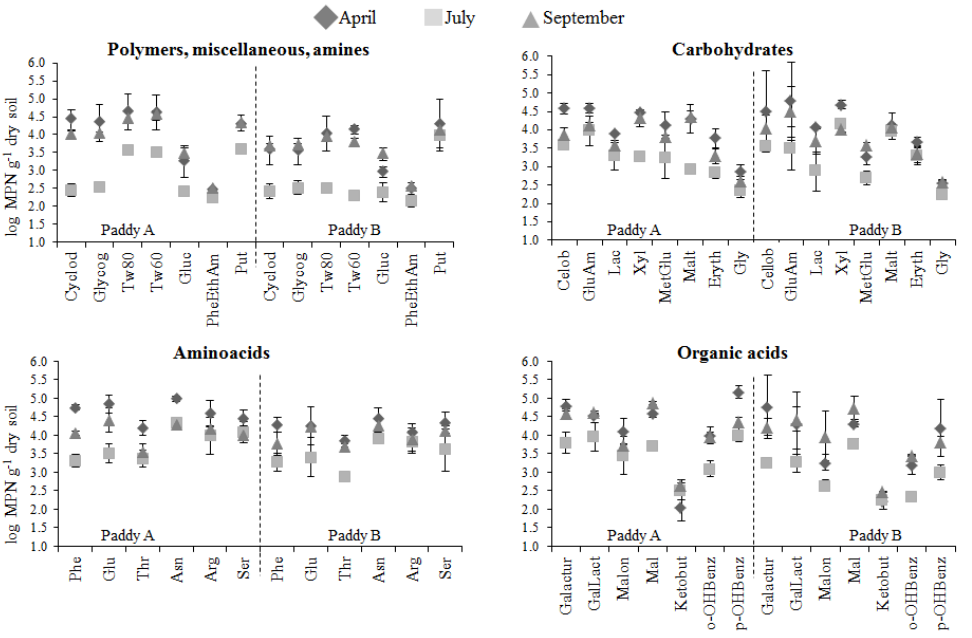

Fig. 6.3 Catabolic profiling data. Density of microorganisms in soil from paddy A and B growing in the tested substrates, over rice cycle, all the values are means ± standard deviation (n=3).

Cyclod: α-cyclodextrin; Glycog: glycogen; Tw80: Tween 80; Tw60: Tween 60; Gluc: α-D-glucose-1-phosphate; PheEthAm: β-phenyl ethylamine; Put: putrescine. Cellob: D-(+) cellobiose; GluAm: *N*-acetyl-D-glucosamine; Lac: α-lactose; Xyl: D-(+) xylose; MetGlu: β-methyl D-glucoside; Malt: maltose; Eryth: i-erythritol; Gly: glyceraldehyde. Phe: L-phenylalanine; Glu: L-glutamic acid; Thr: L-threonine; Asn: L-asparagine; Arg: L-arginine; Ser: L-serine. Galactur: galacturonic acid; GalLact: D-galactonic acid δ-lactone; Malon: malonic acid; Mal: malic acid; Ketobut: α-keto butyric acid; o-OHBenz: o-hydroxybenzoic acid; p-OHBenz: p-hydroxybenzoic acid.

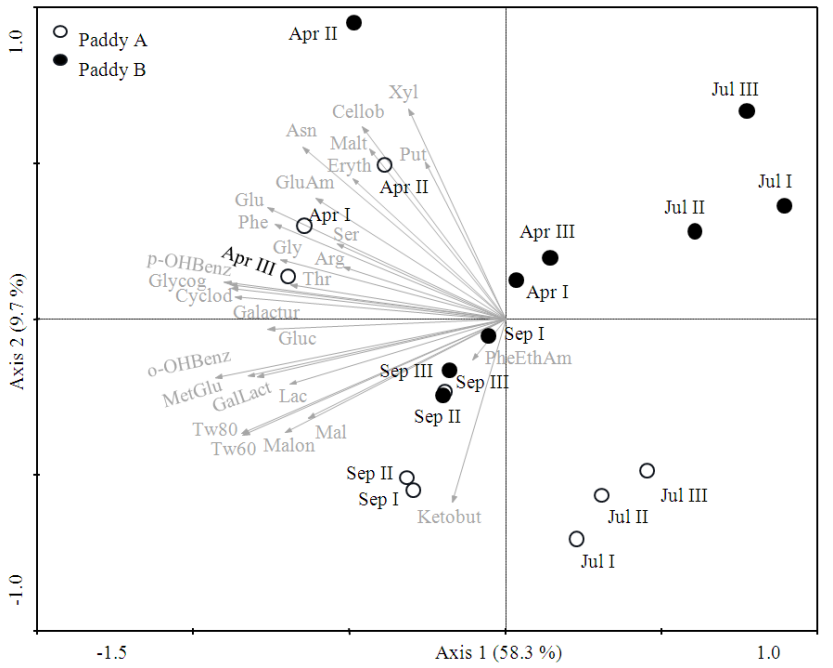

Fig. 6.4 PCA biplot of the catabolic profiling data, density of microorganisms in soil from paddy A and B growing in the tested substrates, over rice cycle.

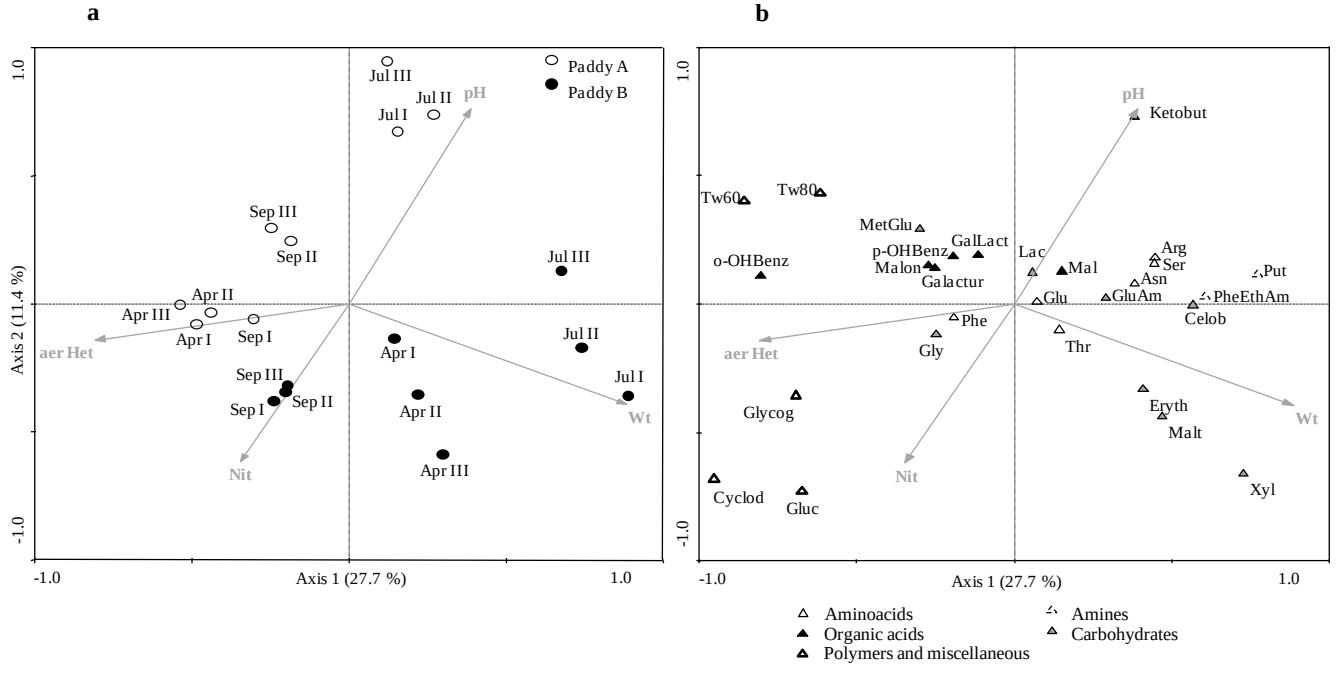

Fig. 6.5 CCA biplot of the variation in catabolic microbial activity (CLPP data) in paddy A and B (total inertia 0.016), over the rice cycle, with arrows indicating the corresponding explanatory variables (water content, abundance of aerobic heterotrophs, pH and total inorganic-N). The species-environmental correlation was 0.946 and 0.876 for axis 1 and 2 respectively. **a**) Representation of samples distribution. **b**) Representation of the C substrates distribution.

6.4.5 Bacterial community structure and composition and parameters correlated with its variation

The pattern of variation of the bacterial community was assessed based on PCA of DGGE patterning (all samples) and 454-pyrosequencing (April and September) (Figs 6.6a and b). These analyses showed consistently the distinction of the structure and composition of the bacterial communities of both paddies, at each stage of the crop rotation.

CCA were performed to assess which were the biotic and abiotic soil parameters most related with the observed variation of the bacterial community structure and composition. Regarding the DGGE analysis, the parameters tested explained 33.4 % of the total variation (Fig. 6.7a). The higher content of available inorganic-P observed in April in paddy B than in paddy A (Table 6.2) was strongly correlated (-0.884 inter-set correlation with axis 1) with the distinction of the bacterial communities of both paddies. On the other hand, the higher abundance of anaerobic diazotrophs in July and September than in April, and of NH_4^+-N produced by mineralization and urease activity in April (-0.670, 0.789, and 0.808 inter-set correlations with axis 2, respectively) contributed to the separation of the samples over the rice cycle.

A similar multivariate analysis based on the 454-pyrosequencing data, explaining a total variation of 29.3%, identified different soil parameters correlated with the variation among the samples (Fig. 6.7b). This may have been due to the fact that the July communities were not included in this analysis. In this case, the higher total C content in paddy B than in paddy A and the highest abundance of aerobic heterotrophs in April, mainly in paddy A, were the parameters that most contributed to the separation of the samples from both paddies (-0.826 and 0.818 inter-set correlations with axis 1, respectively). The aerobic heterotrophs were positively correlated with OTUs affiliated to *Bacteroidetes* (*Flavobacteriales* and *Sphingobacteriales*), *Alphaproteobacteria* (*Caulobacterales*, *Rhizobiales*,

Rhodospirillales and *Sphingomonadales*), and *Actinobacteria* (*Acidimicrobiales* and *Actynomicetales*) (Table 6.2). In contrast, the total C soil content was negatively correlated with these OTUs, and positively with OTUs affiliated to *Bacteroidetes* (*Bacteroidales*), *Chlorobi* and *Chloroflexi* (in particular, *Anaerolineae*) (Table 6.2). On the other hand, the highest abundance of anaerobic diazotrophs in September, and the highest activity of BAA-protease (Table 6.2) in April (-0.914 and 0.898 inter-set correlations with axis 2, respectively) contributed also to distinguish the samples collected in these sampling periods (rice cycle). The abundance of anaerobic diazotrophs was positively related to the OTUs affiliated to *Chloroflexi* (Ellin6529), *Actinobacteria* (*Acidomicrobiales* and *Actinomycetales*), *Alphaproteobacteria* (*Rhizobiales* and *Rhodospirilales*) and *Betaproteobacteria* (Table 6.2). The potential activity of BAA-protease was positively related to OTUs affiliated to *Alphaproteobacteria* (*Rhizobiales)*, *Actinobacteria* (*Actinomycetales*) *Bacteroidetes* (*Sphingobacteriales* and *Bacteroidales*), and *Chloroflexi* (in particular, *Anaerolineae*). Interestingly, these discriminatory parameters were also positively correlated with variations of OTUs affiliated to *Gemmatimonadetes* and *Acidobacteria*, mainly "*Solibactereales*", observed to represent "core" populations, *i.e*., always present although in variable proportions (Lopes et al., submitted).

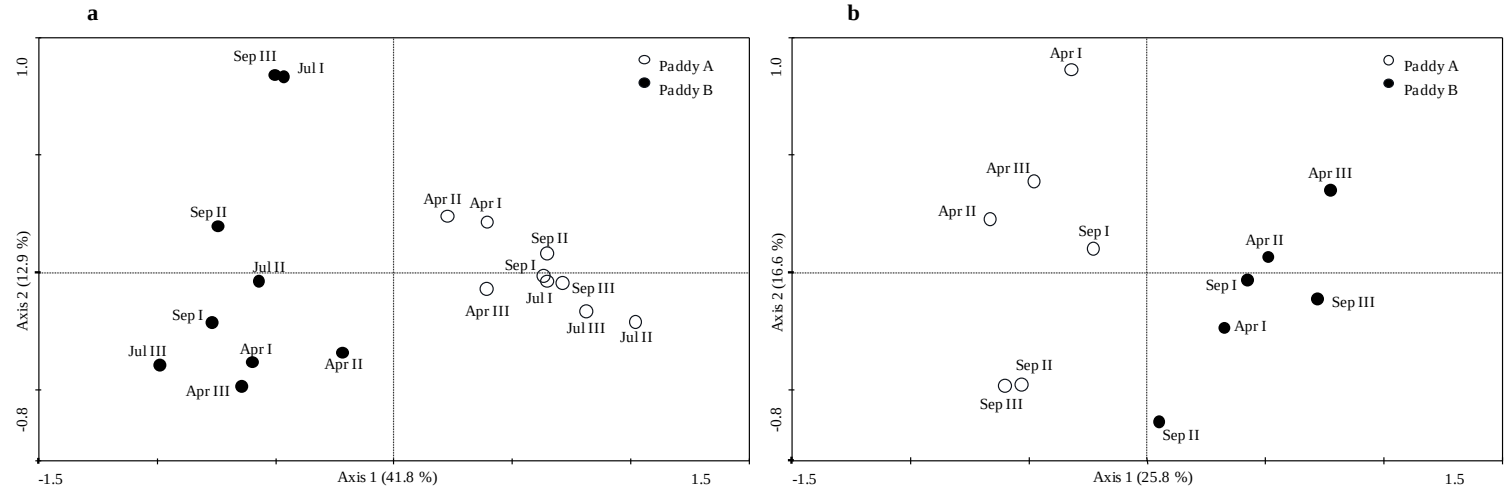

Fig. 6.6 a) PCA biplot of variation in the bacterial community structure and composition (DGGE profiling data) in paddy A and B, over rice cycle. **b**) PCA biplot of variation in the bacterial community structure and composition (pyrosequencing data) in paddy A and B, in April and September.

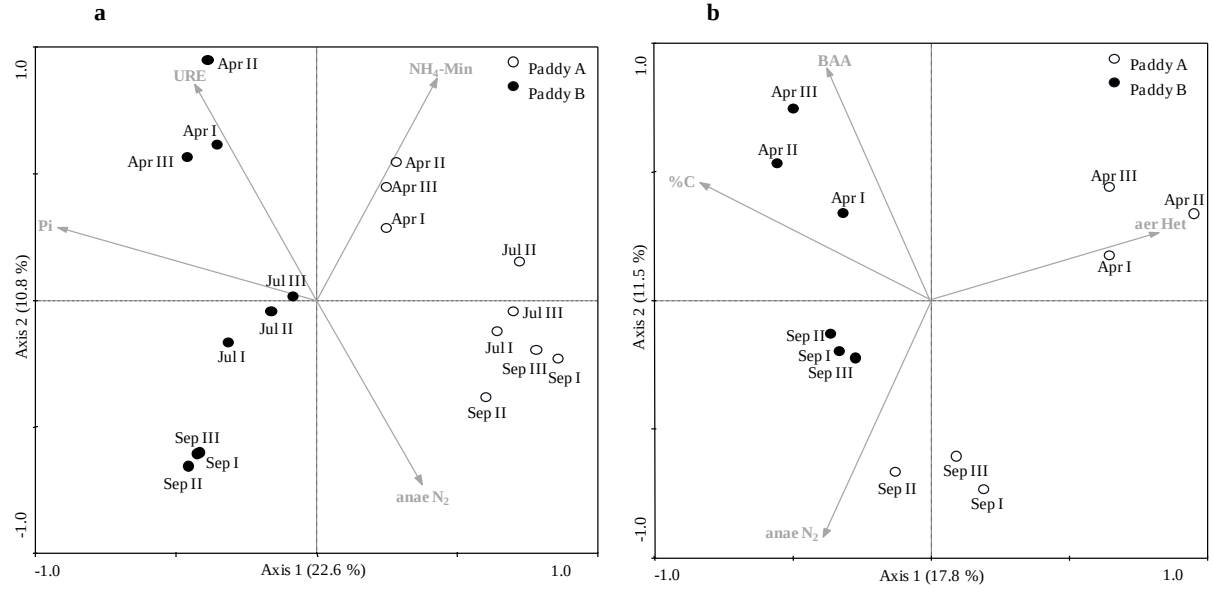

Fig.6.7 CCA biplot of the variation in the structure and composition of bacterial communities of paddy A and B over the rice cycle, with arrows indicating the corresponding explanatory variables. **a**) DGGE profiling data (total inertia 1.504; explanatory variables were available inorganic-P, urease activity, NH_4^+-N mineralization and abundance of anaerobic diazotrophs). The species-environmental correlation was 0.959 and 0.922 for axis 1 and 2, respectively. **b**) pyrosequencing data (samples of April and September; total inertia 0.88; explanatory variables were total C, abundance of aerobic heterotrophs and anaerobic diazotrophs, and BAA-protease activity) The species-environmental correlation was 0.992 and 0.993 for axis 1 and 2 respectively.

Table 6.3 Affiliation number and abundance of OTUs with correlations above 0.4 with axis 1, *i.e.*, with abundance positively correlated with total carbon content (% C) or abundance of aerobic heterotrophs (aer Het) and with axis 2, *i.e.*, with abundance positively correlated with BAA-protease activity (BAA) or abundance of anaerobic diazotrophs (anaer N_2) of the CCA analysis of the 16S rRNA gene pyrosequencing data.

				Number and abundance (%) of OTUs					
Domain	Phylum	Class	Order	A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}	Correlated parameter	Total number of OTUs
Bacteria				**1**	**0**	**1**	**0**	BAA	**1**
	Acidobacteria			**0**	**1**	**3**	**3**	% C	**3**
		Acidobacteria	*Acidobacteriales*	**11**	**10**	**8**	**6**	aer Het	**11**
				6	**7**	**8**	**8**	% C	**8**
				5	**0**	**5**	**3**	BAA	**5**
				2	**4**	**3**	**4**	anaer N_2	**4**
		"Solibacteres"	*"Solibacterales"*	**10**	**6**	**1**	**2**	aer Het	**10**
				6	**11**	**11**	**11**	% C	**11**
				4	**2**	**8**	**3**	BAA	**8**
				3	**9**	**3**	**7**	anaer N_2	**9**
	Actinobacteria			**1**	**0**	**0**	**0**	aer Het	**1**
		Acidimicrobiia	*Acidimicrobiales*	**9**	**4**	**2**	**4**	aer Het	**9**
				1	**1**	**1**	**1**	% C	**1**

Table 6.3 Continued

Domain	Phylum	Class	Order	Number and abundance (%) of OTUs				Correlated parameter	Total number of OTUs
				A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}		
Bacteria	*Actinobacteria*	*Acidimicrobiia*	*Acidimicrobiales*	**0**	**0**	**1**	**0**	BAA	**1**
				1	**6**	**1**	**4**	anaer N_2	**6**
		Actinobacteria	*Actinomycetales*	**11**	**2**	**2**	**1**	aer Het	**11**
				0	**1**	**1**	**1**	% C	**1**
				1	**1**	**2**	**1**	BAA	**2**
				1	**4**	**2**	**3**	anaer N_2	**4**
	Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	**4**	**3**	**1**	**2**	aer Het	**4**
				0	**0**	**1**	**0**	BAA	**1**
		Sphingobacteriia	*Sphingobacteriales*	**47**	**17**	**10**	**5**	aer Het	**47**
				1	**1**	**1**	**1**	% C	**1**
				3	**1**	**5**	**2**	BAA	**5**
				0	**4**	**0**	**2**	anaer N_2	**4**
		Bacteroidia	*Bacteroidales*	**2**	**2**	**2**	**2**	% C	**2**
				2	**0**	**4**	**0**	BAA	**4**
				0	**1**	**0**	**1**	anaer N2	**1**
	Chlorobi			**2**	**3**	**5**	**5**	% C	**5**

Table 6.3 Continued

				Number and abundance (%) of OTUs					
Domain	Phylum	Class	Order	A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}	Correlated parameter	Total number of OTUs
Bacteria	*Chlorobi*			**0**	**0**	**2**	**0**	BAA	**2**
	Chloroflexi			**1**	**0**	**0**	**0**	aer Het	**1**
				1	**0**	**1**	**0**	BAA	**1**
				1	**4**	**0**	**2**	anaer N_2	**4**
		Anaerolineae		**2**	**0**	**0**	**0**	aer Het	**2**
				2	**5**	**8**	**8**	% C	**8**
				0	**0**	**2**	**0**	BAA	**2**
				0	**2**	**0**	**0**	anaer N_2	**2**
			Anaerolineales	**0**	**1**	**2**	**2**	% C	**2**
				0	**0**	**2**	**0**	BAA	**2**
				0	**1**	**0**	**0**	anaer N2	**1**
		"Ellin6529"		**10**	**5**	**3**	**3**	aer Het	**11**
				0	**1**	**1**	**1**	% C	**1**
				1	**6**	**1**	**3**	anaer N2	**6**
	Firmicutes	*Clostridia*		**1**	**1**	**0**	**0**	aer Het	**1**
			Clostridiales	**0**	**3**	**0**	**0**	anaer N2	**3**

Table 6.3 Continued

Domain	Phylum	Class	Order	Number and abundance (%) of OTUs				Correlated parameter	Total number of OTUs
				A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}		
Bacteria	*Gemmatimonadetes*	*"Gemm-1"*		**4**	**2**	**1**	**1**	aer Het	**4**
				2	**2**	**1**	**2**	anaer N_2	**2**
		Gemmatimonadetes		**3**	**1**	**1**	**0**	aer Het	**3**
				0	**1**	**1**	**1**	% C	**1**
				2	**1**	**2**	**2**	BAA	**2**
				1	**2**	**1**	**1**	anaer N_2	**2**
	Proteobacteria	*Alphaproteobacteria*		**10**	**3**	**2**	**2**	aer Het	**10**
				1	**1**	**1**	**1**	BAA	**1**
				1	**1**	**1**	**1**	anaer N_2	**1**
			Caulobacterales	**5**	**3**	**1**	**2**	aer Het	**5**
				1	**1**	**1**	**1**	BAA	**1**
			Rhizobiales	**10**	**7**	**4**	**5**	aer Het	**10**
				2	**2**	**3**	**3**	% C	**3**
				6	**1**	**6**	**3**	BAA	**6**
				1	**2**	**1**	**2**	anaer N2	**2**
			Rhodobacterales	**2**	**1**	**1**	**1**	aer Het	**2**

Table 6.3 Continued

Domain	Phylum	Class	Order	Number and abundance (%) of OTUs				Correlated parameter	Total number of OTUs
				A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}		
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	**8**	**5**	**3**	**2**	aer Het	**8**
				0	**1**	**1**	**1**	% C	**1**
				1	**0**	**1**	**0**	BAA	**1**
				0	**3**	**1**	**1**	anaer N_2	**3**
			Rickettsiales	**1**	**0**	**0**	**0**	aer Het	**1**
				1	**3**	**1**	**1**	anaer N_2	**3**
			Sphingomonadales	**16**	**9**	**5**	**6**	aer Het	**16**
				1	**0**	**1**	**0**	BAA	**1**
		Betaproteobacteria		**1**	**0**	**0**	**0**	aer Het	**1**
				0	**3**	**0**	**1**	anaer N2	**3**
			Burkholderiales	**1**	**0**	**0**	**0**	aer Het	**1**
				1	**0**	**1**	**0**	BAA	**1**
				0	**1**	**0**	**0**	anaer N2	**1**
		Deltaproteobacteria		**1**	**2**	**0**	**0**	anaer N2	**2**
			Bdellovibrionales	**0**	**1**	**0**	**0**	anaer N2	**1**
			Desulfuromonadales	**0**	**1**	**0**	**0**	anaer N2	**1**

Table 6.3 Continued

Domain	Phylum	Class	Order	Number and abundance (%) of OTUs				Correlated parameter	Total number of OTUs
				A_{Apr}	A_{Sep}	B_{Apr}	B_{Sep}		
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	**2**	**0**	**0**	**0**	aer Het	**2**
				0	**1**	**0**	**0**	anaer N_2	**1**
		Gammaproteobacteria	*Methylococcales*	**0**	**1**	**0**	**0**	anaer N_2	**1**
	Verrucomicrobia	*Opitutae*	*"Cerasicoccales"*	**0**	**0**	**1**	**0**	BAA	**1**

abundance (sum the % of individual OTUs)= 0

abundance (sum the % of individual OTUs) > 0 and < 0.1

abundance (sum the % of individual OTUs) > 0.1 and < 0.5

abundance (sum the % of individual OTUs) > 0.5 and < 1

abundance (sum the % of individual OTUs) > 1

6.5 Discussion

In a previous study based on 16S rRNA gene 454-pyrosequencing analysis, it was observed that the stage of alfalfa-rice crop rotation and the rice cropping cycle influenced the structure and composition of the bacterial community (Lopes et al., submitted). The current study aimed to get further insights into the factors that may be responsible for/or resultant of such community variations, regarding microbial activity and external factors, over the rice cycle or crop rotation stage. The combination of 454-pyrosequencing, DGGE and cultivation based methods seemed the best approach to fulfil the proposed objectives. The 454-pyrosequencing and DGGE community analyses based on the 16S rRNA gene sequence were in good agreement and the measurement of the functional activities of the communities gave an important input for the interpretation of the culture-independent methods. As expected, bacterial community variations could be correlated with the catabolic profiles, enzymatic activity and abiotic parameters. Different factors seem to be determinant in shaping the microbial communities over the rice cycle or when the paddies are under different stages of crop rotation. Correlations between the availability of nutrients and oxygen, physiological microbial groups and bacterial diversity were clear and well sustained.

6.5.1 Variations over the rice cycle

Culture dependent and culture independent methods allowed the distinction of the structure and composition of the bacterial communities of April from those of the other periods, in samples A and B (Figs 6.1b and 6.6). At this sampling period, significant positive correlation between the aerobic heterotrophic population and proteolytic activity and the 16S rRNA gene-based bacterial community structure and composition were observed (Figs 6.7 a or b). According to the multivariate analysis, members related with *Bacteroidetes-Sphingobacteriales/Bacteroidales*, *Alphaproteobacteria-Rhizobiales*, *Actinobacteria* (*Actinomycetales*) and

Chloroflexi-Anaerolineae could have been involved in the soil proteolytic activity. Excepting the members of the last group, which were only present in B_{Apr}, all the other, although present in both paddies, were, in general, in higher number in B_{Apr} than in A_{Apr}. These differences may explain the higher proteolytic activity determined in paddy B than in paddy A. The activities of both proteases (casein and BAA) and urease were associated with the microbial coefficient (qCO_2), probably due to the intense microbial activity occurring in April, eventually contributing to the transformation of complex into simpler organic N-compounds before flooding.

In addition, in the subsequent periods fixed atmospheric N_2 was likely incorporated in the soil N pool. Indeed, the soils NH_4^+-N content in July and September was higher than in April (Table 6.2). On the other hand, in July and September, strong positive correlations between the cultivable diazotrophic populations and OTUs affiliated to taxonomic groups described as comprising N_2 fixing members (*Chloroflexi*-Ellin6529, *Actinobacteria-Acidomicrobiales/Actinomycetales*, *Betaproteobacteria* and *Alphaproteobacteria-Rhizobiales* /*Rhodospirilales*) (Dos Santos et al. 2012; Gaby and Buckley 2011) (Figs 6.1b and 7), suggest the enrichment of paddy soils in NH_4^+ from N_2. Indeed, this transformation represents the most important source of N for rice plants (Arima 1978; Arima and Kumazawa 1977), explaining its intensification only when rice plants were present. This hypothesis is supported by other studies which report the increase of diazotrophic populations over the rice plants growth towards maturation, in particular in rice rhizosphere (Ikenaga et al. 2003; Knief et al. 2012; Watanabe et al. 1979). Thus, as suggested before (Kundu and Ladha, 1995), diazotrophs may have contributed to replenish the total N content of rice paddies and consequently improved the NH_4^+ bioavailability. On the other hand, the flooding conditions maintained over rice growth (in particular in July), could have decreased the loss of ammonia via volatilization (Overrein and Moe 1967), maintaining high levels of NH_4^+-N in paddy fields.

These kind of seasonal variations find good support on the rice crop management, since in April the paddies were still under upland conditions, and thus, with a higher degree of oxygenation than in the later sampling periods. Indeed, it is well described that the rapid oxygen depletion caused by flooding induces changes on the soil microbial populations of rice paddies (Kikuchi et al. 2007; Noll et al. 2005; Shrestha et al. 2009), which are probably correlated with functional adjustments of the communities. For instance, Shrestha et al. (2009) observed a higher abundance of transcripts related to carbohydrates, amino-acids and lipids metabolism under oxic than under anoxic conditions in paddy soils.

6.5.2 Variations related with the crop rotation stage

The crop rotation stage imposed significant alterations in the structure and composition of the bulk soil communities, which were mainly related with differences in the total C- and available inorganic-P contents (Fig. 6.7). Phosphorus availability has been described as a major driver of differences on bacterial community structure and composition among soils with similar pH (Kuramae et al. 2011). In addition, soil C content and availability have been described as important factors shaping soil bacterial communities (Ausec et al. 2009; Fierer et al. 2007a). In spite of the differences in the bacterial community revealed by culture independent techniques, cultivation methods showed mild variations associated with the crop rotation stage. Indeed, although the bulk soil cultivable populations corresponding to the samples of 3rd and 4th years of the rotation cycle were distinguishable, clustered mainly by sampling period (Fig. 6.1b).

In general, it was in April that the effect of the crop rotation stage was more notorious. It is important to remember that in April both paddies were being prepared for rice cultivation, but whereas paddy A (3rd year of the rotation) had been cultivated with alfalfa for two years, paddy B (4th year of the rotation) was planted with rice in the previous year, and was organically and inorganically amended during field preparation. At this period, not only 16S rRNA gene

community patterning but also the catabolic profiling allowed a clear distinction of the microbial populations of both paddies. Paddy A contained the highest number of organisms growing aerobically on the tested single C sources (Fig. 6.4). The use of complex substrates (o-hydroxybenzoic acid, tween 60, α-cyclodextrin, and glycogen) was positively correlated with the highest number of aerobic heterotrophs in A_{Apr} (Fig. 6.5). Consistent with this observation, was the strong correlation between the aerobic populations and the bacterial community structure and composition of paddy A in April (Fig. 6.7b). Indeed, the high abundance of aerobic heterotrophs in this paddy and period was correlated with different OTUs affiliated to groups comprising presumable aerobic organotrophs, such as *Bacteroidetes* (*Flavobacteriales* and *Sphingobacteriales*), *Alphaproteobacteria* (*Caulobacterales*) and *Actinobacteria* (*Actinomycetales*). Such organisms, which have been described as being involved in the degradation of complex organic matter (e.g., cellulose, hemicellulose and lignin) (Berg and McClaugherty 2008; DeAngelis et al. 2011), may have contributed to the degradation of alfalfa debris (in particular, roots) present in aerated bulk soil in April, in paddy A.

Compared to paddy A, in April, paddy B presented higher water content and a lower number of aerobes using single C substrates (Fig. 6.4). On the other hand, OTUs of presumably anaerobic organotrophic members (*Bacteroidetes-Bacteroidales*, *Chlorobi* and *Chloroflexi-Anaerolineae*) were strongly positively correlated with the soil total C content and were determinant for distinguishing the bacterial community structure and composition of paddy B. These coincidences may suggest that the higher water content in paddy B was associated with a lower oxygenation of the soil. Thus, it could be hypothesized that the effectiveness in restoring the soil oxygenation may be limited in the last year (4^{th}) of the crop rotation.

Interestingly, not only the number, but also the type of single C sources preferentially used (amino acids, amines and carbohydrates) was different in samples B_{Apr} and A_{Apr}, with the catabolic profile of B_{Apr} closer to those of A_{Jul} and

B_{Jul} (Fig. 6.5). Therefore, it can be hypothesized that the higher water content in paddy B may have been unfavourable for the degradation of more recalcitrant carbon sources, such as lignin (enriched in phenolic compounds), which biodegradation is particularly dependent on the oxygen availability (Berg and McClaugherty 2008). An additional interpretation is that the available organic compounds in April in paddy A were distinct from those in paddy B. Considering such hypothesis, the organic compounds available in April in paddy B, would be similar to those found in July in both paddies, *i.e.*, those probably resultant from rice root exudates, which highest production occurs near the maximum tillering stage (July) (Ikenaga et al. 2003). In fact, in April, paddy B, but not in paddy A, could have rice residues resultant from the incorporation in soil of rice stubble after harvesting in the previous year, and contained an organic amendment. The abundance of organic compounds, derived from both sources, may have stimulated the intense proteolytic activity in paddy B, explaining the availability of N-containing organic compounds, such as amino acids and/or amines supporting the growth of aerobic populations in B_{Apr} samples.

In paddy B in April, probably due to the low oxygen availability referred to above, the anaerobic degradation of N-containing organic compounds was also relevant. Therefore, not only presumable aerobic (*Alphaproteobacteria-Rhizobiales*, *Bacteroidetes-Sphingobacteriales*, *Actinobacteria-Actinomycetales*), but also anaerobic (*Bacteroidetes-Bacteroidales*, *Chloroflexi-Anaerolineae*) bacteria may have been involved in the degradation of N-containing organic compounds. Indeed, BAA-protease activity was strongly positively correlated with those lineages. The anaerobic decomposition of organic matter in paddy soils may be disadvantageous since can lead to the accumulation of reduced compounds, capable of hampering rice plants N-uptake (Kundu and Ladha, 1995). Such a phenomenon may have contributed for the lower rice yield of paddy B compared to A. Therefore, rice-farming techniques favouring the maintenance of catabolic active aerobic populations are advisable.

Chapter 7

General Discussion

The development and application of culture-independent methods (e.g., 16S rRNA gene clone libraries), in the 1980's, showed that the bacterial diversity is far beyond what, so far, is identified and available in the culture collections (Janssen 2006; Keller and Zengler 2004; Rappé and Giovannoni 2003). Indeed, while in 1987, Woese proposed 11 major groups or bacterial lineages, nowadays 30 phyla are known and more than 23 candidate phyla have been proposed based on 16S rRNA gene sequences analysis (e.g., TM7, OP3, OP11) (Keller and Zengler 2004; Rappé and Giovannoni 2003). Culture-independent methods not only revealed that only a very small fraction of the bacterial diversity is known, but also demonstrated that some organisms, which were frequently recovered using cultivation methods, are not the most representative in the environment. Regarding soil microbiota, cultivable bacteria, such as members of the genera *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Streptomyces*, considered very important community members, were, after all, not representative of the total soil diversity (Janssen 2006). In the last decade, the high throughput sequencing techniques (e.g., 454-pyrosequencing) (Mardis 2008) strengthen the idea that much of the bacterial diversity is still unknown, not only at the phylum level, but also beneath. Indeed, even phyla well represented in bacterial diversity studies, such as *Proteobacteria*, still contain numerous unknown lineages. Moreover, given the capability of these techniques to analyse simultaneously a large number of samples, some phyla, considered rare few years ago, such as *Acidobacteria* and *Gemmatimonadetes*, are now considered phylogenetically diverse and ubiquitous in soils (in particular, *Acidobacteria*) (DeBruyn et al. 2011; Jones et al. 2009; Kielak et al. 2008b). Thus, currently, despite the known heterogeneity and diversity of soil matrices, the phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Firmicutes* are considered the most abundant in soil habitats (Eilers et al. 2012; Janssen 2006; Roesch et al. 2007). Nevertheless, the ecology of soil microbiota is rather complex and the occurrence of other groups as well as the abundance of each of those lineages vary with the biotic and abiotic conditions prevailing in each soil ecosystem (Ausec et

al. 2009; Kuramae et al. 2011; Kuramae et al. 2012; Lauber et al. 2009; Ulrich and Becker 2006) (Chapters 5 and 6).

The agriculture activity has been reported to induce changes in, for example, the physicochemical soil properties (e.g., lower organic matter content when compared with uncropped soils) (Haynes and Tregurtha 1999; Jangid et al. 2011; Trasar-Cepeda et al. 2008), on the structure and composition of bacterial communities (Crecchio et al. 2004; Jangid et al. 2011; Roesch et al. 2007; Stark et al. 2008; van Diepeningen et al. 2006), and also on the activity of bacterial communities (Degens et al. 2001; Nsabimana et al. 2004). The influence of agriculture management on the soil bacterial communities was explored at different levels and using different tools in Chapters 3, 5 and 6. It was observed that the continuous application of synthetic plant protectors and fertilizers in rice cultivation under conventional farming induced changes on the bacterial community structure and composition of paddy soils (Chapter 3). On the other hand, the presence of rice plants (cropped/uncropped), the type of plant debris (stage of rotation), and growth phase of the rice plants (annual rice cycle) were correlated with variations on the structure and composition of the bacterial communities of paddy soils and their biochemical activities (Chapters 5 and 6). Arguably, other factors may be involved in the observed changes. Indeed, seasonal changes on the bacterial community composition of soils under organic farming, irrespectively of rice monoculture (Chapter 3) or rice rotation with alfalfa (Chapters 5 and 6), were observed in the present study, and were also described by other authors (Chen et al. 2012; Kikuchi et al. 2007; Lüdemann et al. 2000; Noll et al. 2005). However, curiously, such changes were not observed under conventional management (Chapter 3). This may suggest that this pesticide attenuated possible variations on the type and/or abundance of the organisms otherwise observed over the annual rice cycle.

Among the variables studied in soils under organic farming, the presence of rice plants stood out as the major factor contributing to shape the structure and composition of bulk soil bacterial communities. The absence of rice crop promoted

an increase of the bacterial diversity. Similar conclusions were previously retrieved from studies performed in lysimeters with unplanted and planted (grass and forb species) upland soils (Zul et al. 2007). These studies demonstrate that plants growth shapes the structure and composition of bacterial communities not only on the rhizosphere, as previously reported (Berg and Smalla 2009; Ikenaga et al. 2003; Lu et al. 2006) but also on the bulk soil.

The bacterial community is extremely important to the maintenance of soil functional activity due to the role of the organisms on soil biogeochemical cycles (Emerson et al. 2010; Falkowski et al. 2008; Gaby and Buckley 2011; Ghosh and Dam 2009; Hanson and Hanson 1996; Hohmann-Marriott and Blankenship 2011; Zumft 1997), soil architecture (Maier and Pepper 2009), interaction with plants (Gomes et al. 2010; Little et al. 2008) and capacity to respond to induced stresses (Degens et al. 2001). Studies on the soil bacterial community composition and structure are, thus, important. Studies based on the 16S rRNA gene sequence analyses are very popular in this respect. This phylogenetic marker gives an indication of the diversity in the soil, and since activity is not inferred using this tool, the relative abundance offers a perspective of the most relevant lineages under specific conditions or at a given time. Therefore, even if part of the identified organisms is not active at a given period, they can be stimulated and become active under particular (yet, important) situations. This rationale explains the successional variations in paddy soils bacterial communities, reported in the rhizosphere (Ikenaga et al. 2003; Lu et al. 2006), associated with plant debris degradation (Akasaka et al. 2003; Asari et al. 2007; Rui et al. 2009), or under oxygenation gradients (Lüdemann et al. 2000; Noll et al. 2005; Shrestha et al. 2007). It is not mandatory that these transitions involve closely related bacteria, since important metabolic activities (e.g. nitrogen fixation or polymer degradation) are phylogenetically widespread (Berg and McClaugherty 2008; Buckley et al. 2007; Dos Santos et al. 2012; Eichorst and Kuske 2012), or can be acquired by horizontal gene transfer (Dröge et al. 1999; Ma et al. 2006). Indeed, the same function can be conducted by different lineages, depending on the environmental conditions (e.g.,

temperature, availability of a particular nutrient) (Liu and Conrad 2011; Rui et al. 2009; Scheid et al. 2004; Shi et al. 2011). This observation clearly emphasizes the importance of environmental conditions in ecological succession of bacterial communities. In the present study, bacterial community succession and functional redundancy were demonstrated in different occasions. The first evidence of functional redundancy was given by the similar functional microbial activity observed over the annual rice cycle under both agriculture managements (conventional and organic, respectively), despite the differences on the bacterial community structure and composition (Chapter 3). The ability of autochthonous microbial communities from non contaminated soil to degrade the herbicide molinate (Chapter 4) is also an example. Although molinate hydrolysis in soil was not via molinate hydrolase (MolA) activity (Duarte et al. 2011), suggesting the absence of indigenous bacteria related to *Gulosibacter molinativorax*, other organisms were capable of molinate degradation. These results suggest the existence of a diverse metabolic pool, which allows the soil bacterial communities to respond to different environmental stimuli.

The effect of different pesticides on the microbial community structure and activities may be transient (Chen et al. 2009; Das and Mukherjee 2000; El-Ghamry et al. 2001; Saison et al. 2009) (Chapter 4). However, the continuous application of these compounds may induce long-lasting changes in the bacterial community composition of paddy soils, though microbial functional activity is not affected, as was revealed in the present study (Chapter 3). The effect of the rotation stage (different upland crops in rotation with rice) has been described to shape the bacterial communities in paddy fields (Chen et al. 2012; Xuan et al. 2012), although no detailed characterization of the bacterial communities was available. To the best of our knowledge this study is the first reporting a thorough characterization of the bacterial communities under an alfalfa-rice rotation system. Furthermore, the present study goes further demonstrating that the change in the ratio and composition of aerobic and anaerobic heterotrophs may be important in shaping the paddy microbial responses to specific nutrients (changing its catabolic

profiling) (Chapters 5 and 6). These changes may be related to the decrease in rice productivity observed from the 1st to the 2nd year of rice production (respectively 3rd and 4th years of the crop rotation). The higher water content (in paddy B in the 4th year of the crop rotation) and low efficiency in soil aeration between rice crops, under fallow period, may have induced less oxidative conditions in the 2nd year of rice production than in the 1st. This condition could have been associated with the accumulation of reduced compounds which strongly inhibit the N-uptake by plants (Berg and McClaugherty 2008; Kundu and Ladha 1995). These findings support the recommendation of rice-farming techniques that favour the maintenance of the catabolic active of aerobic populations.

Chapter 8

Main Conclusions

The main conclusions of this work can be summarized as follows:

- Under organic farming, including under alfalfa-rice crop rotation, were observed changes of the bacterial community structure, composition and function over the annual rice cycle. However, under conventional farming, only changes on the functional activity were observed. These results demonstrate soil microbial functional redundancy;

- The presence (growth) of rice crop, more than the crop rotation stage or the period of the rice cycle, influenced the structure and composition of the bulk soil bacterial community. In comparison with cropped soils, the uncropped area presented higher bacterial diversity, with the raise of lineages such as *Chloroflexi* ("Ellin 6529"), *Acidobacteria*-2, AD3, and *Nostocaceae*, outcompeted in cropped soils*;*

- Over the rice cycle, the major microbial functions of the bulk paddy soil were i) the organic matter degradation (microbial coefficient and proteolytic activity), intense before rice seeding, *i.e.*, under upland conditions, and ii) N_2 fixing, strongly associated with rice plants growth, *i.e.*, under flooded conditions. The former was associated with *Sphingobacteriales*, *Rhizobiales*, *Actinomycetales*, *Bacteroidales* and *Anaerolineae*, whereas the later was related with high abundance of cultivable diazothrophs and *Chloroflexi*-Ellin6529, *Actinobacteria-Acidomicrobiales/Actinomycetales*, *Betaproteobacteria* and *Alphaproteobacteria-Rhizobiales*/*Rhodospirilales* lineages. OTUs affiliated to *Gemmatimonadetes* and *Acidobacteria*, in particular "*Solibacteres*", were probably involved in both functions;

- In addition, over the rice cycle there was an increase of members affiliated to *Nitrospira*;

- Crop rotation stage affected the ratio of aerobic:anaerobic heterotrophic cultivable populations, mainly before rice seeding, presumably favouring the development of lineages such as *Caulobacterales*, *Sphingobacteriales*,

Flavobacteriales and *Actinomycetales*, more notorious in the first year of rice cultivation. In the 2nd year of rice cropping was observed a higher content of water, total C and available inorganic-P, which in combination of the organic amendments and/or rice debris, may have supported the proliferation of presumable anaerobic members *Bacteroidales*, *Chlorobi* and *Anaerolinea*, but not of cultivable aerobic heterotrophs;

- Different aerobic catabolic profiles of soils were observed at the 1st and the 2nd year of rice cultivation, presumably due to the dynamics of the microbial communities. The high abundance of aerobic heterotrophs was correlated with the degradation of complex nutrients, while low levels of aerobic heterotrophs were correlated with the degradation of amino acids, amines and sugars;

- The most abundant phyla in organically farmed paddy bulk soil under alfalfa-rice crop rotation system were *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria* and *Bacteroidetes*; and the most abundant families were *Sphingomonadaceae*, "*Solibacteraceae*" and "*Koriobacteraceae*";

- Molinate was removed from contaminated rice paddy soils using both natural attenuation and bio-augmentation methods;

- A bioaugmentation process involving mixed culture DC was demonstrated as a feasible solution to remediate soils contaminated with molinate, since the autochthonous bacterial community was not disturbed and the removal of the contaminant was faster/more extensive than that obtained with the autochthonous community.

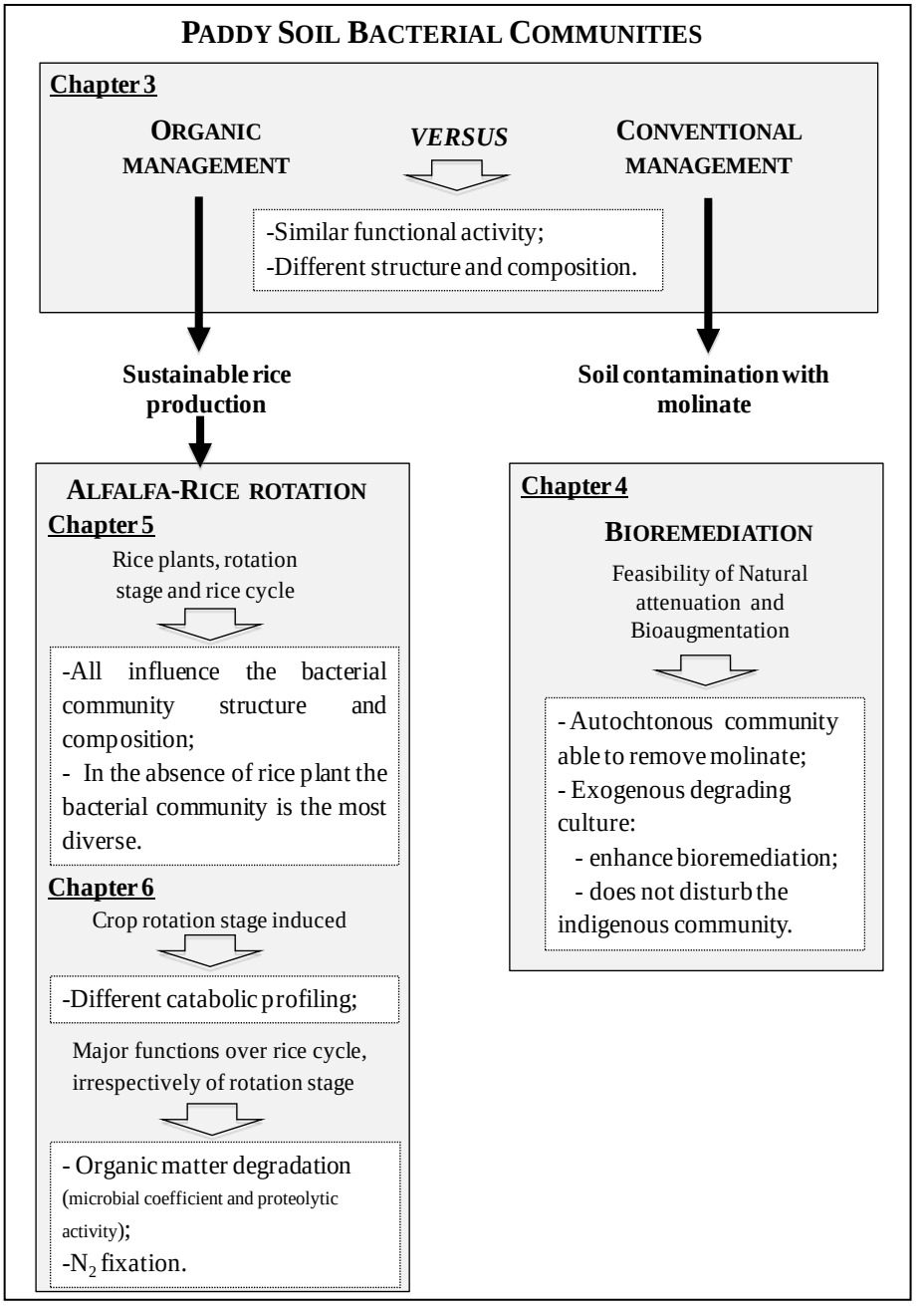

Fig. 8.1 Scheme of the major conclusions of the present study.

Chapter 9

Proposal for Additional and Future Work

The present study gave important clues about the effect of different rice production managements on the bacterial structure and composition and microbial functional activity, in paddy soils. In particular, brought some glimpses about the bacterial lineages inhabiting paddy soils under alfalfa-rice rotation system, the changes in the bacterial community and structure and the correlation of some lineages with biological activities occurring before and after flooding. Yet, some other questions that should be addressed in further studies have emerged in parallel with the conclusions:

- An increase in the abundance of the diazotrophic population over the rice cycle independently of the stage of the alfalfa-rice rotation stage was observed in the present study. Additional research is necessary to conclude if the stage of the alfalfa-rice rotation influenced the diversity and abundance of the diazotrophic populations. This study is undergoing.

- The present study demonstrated that rice cropping induces significant changes in the diversity of bacterial communities. However, it remains unclear if such changes were accompanied by alterations of the microbial activities in the uncropped soils. This study is undergoing.

- Given the important role of some microbial lineages, such as *Archaea* or anammox, on the biogeochemical cycles (e.g., ammonia oxidation and methanogenesis), the assessment of the abundance and activity of these organisms using targeted approaches (e.g. qPCR) would bring additional information on the influence of the agriculture management on these soil activities.

- In spite of the comprehensive snapshot of the major activities occurring in paddy soils under alfalfa rice rotation system and over the annual rice cycle, many other soil functions were not studied. A transcriptomic approach assessing the major metabolic activities occurring over rice cycle and at the different stages of the crop rotation would be helpful to have a thorough characterization of the role of some members in the soil functional activity. Such science based data would be crucial for a sustainable rice production.

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