INFLUENCE OF GRASSLAND MANAGEMENT AND HERBIVORY ON DIVERSITY AND ECOLOGY OF PLANT-ASSOCIATED BACTERIAL COMMUNITIES

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“We have to remember that what we observe is not nature itself, but nature exposed to our method of questioning”

Werner Heisenberg

(German physicist and Noble Price laureate)

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ABBREVIATIONS

ACE	ACE richness estimator
ANOVA	Analysis of Variance
a.s.l.	above sea line
BLAST	Basic Local Alignment Search Tool
BLASTN	BLAST search using a nucleotide query
bp	base pairs
°C	degree Celsius
ca.	circa
cm	centimeter
D.	*Dactylis*
DEPC	Diethylpyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E	East
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia, for example
EU	European Union
EU-27	Member of the European Union
F.	*Festuca*
Fig.	figure
Figs.	figures
g	gram
GrassMan	Grassland Management Experiment
h	hour(s)
ha	hectare
i.e.	id est, that is
k	kilo
L	litre
L.	*Lolium*
m	meter

µ	micro
min	minute
mm	millimetre
N	North
NCBI	National Centre for Biotechnology Information
$n_{max.}$	maximal OTU number
NPK	Fertilizer containing Nitrogen, Phosphorous, and Potassium
nt	nucleotides
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PLFA	Phospholipid Fatty Acid
pH	power of hydrogen
QIIME	Quantitive Insights Into Microbial Ecology
rDNA	DNA coding for ribosomal DNA
PCoA	Principal Coordinate Analysis
PGPR	Plant Growth Promoting Rhizobacteria
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	second
SSU	Small Subunit of the Ribosome
Tab.	table
TAE	tris-acetate-EDTA
Taq	*Thermus aquaticus*
TE	Tris EDTA
Tr.	Treatment
Tris	tris(hydroxymethyl)aminomeethane
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USA	United States of America
UV	ultraviolet
V	volt
vs.	versus

yr	year

Abbreviations for nucleotides

A	adenine
C	cytosine
G	guanine
R	purines (adenine or guanine)
T	thymine (5-methyluracil)
Y	pyrimidines (cytosine, thymine or uracil)

CHAPTER A

SUMMARY

1 SUMMARY

In the last years, plant-associated bacterial communities caught the attention of investigators due to their importance for both plant health and the environmental balance. Despite the increasing number of studies, there is still a knowledge gap about the influence of management regimes on the diversity of plant-associated bacteria in grassland ecosystems.

In this study, we gained new and interesting insights into the diversity of plant-associated bacteria in grassland ecosystems. All investigations in this study were carried out in the same area, the GrassMan experimental field in the Solling Uplands, central Germany. The GrassMan project was set up in 2008. It was conducted in a matrix of meadow plots at a permanent grassland site. The full-factorial design of GrassMan included two mowing frequencies (mowing once per year in July vs. mowing thrice per year in May, July, and September) and two fertilization treatments (no vs. fertilization with NPK). A third factor, the gradient of species richness, was manipulated by selective herbicide applications targeting either dicots or monocots.

The first aim of this study was to investigate the effect of the different mowing and fertilization regimes onto the bacterial endophytic community in three grass species, *Festuca rubra*, *Lolium perenne*, and *Dactylis glomerata*, respectively. Therefore, tiller samples were taken from the dicot-reduced plots in September 2010 as well as in April, July, and September 2011. Total DNA was extracted from the collected samples and subjected to 16S rRNA gene PCRs. Community structures were assessed by DGGE-based analysis of the generated PCR products.

We found differences in bacterial endophyte community structures with respect to the grassland management regimes investigated. While fertilizer application had a high impact onto endophytic diversity in both *F. rubra* and *L. perenne*, the endophytic community structure in *D. glomerata* was not influenced by this management regime. Moreover, tillers of *L. perenne* derived from unfertilized plots grouped in distinct clusters indicating a more similar bacterial community composition in these plots when analyzing for the influence of the mowing treatment.

We also recorded a strong seasonal effect on community composition. As a consequence, both the season and the host plant have to be regarded in further studies as they might alter the effects of different grassland management regimes on endophytic bacterial community structures.

The second aim of this study was to investigate the effect of above-ground herbivory on the bacterial community composition in the rhizosphere. Therefore, a lysimeter experiment was established in autumn 2010. Following a two-week exposure to herbivory by grasshoppers and snails, soil samples were collected from the lysimeters in summer 2011. To gain insights into the composition of the plant-associated bacterial communities in the rhizosphere, total DNA was extracted from the collected samples and subjected to 16S rRNA gene PCRs. Community structure were assessed either by DGGE analysis or pyrosequencing of the obtained PCR products.

Whereas herbivory did not seem to affect the bacterial richness, slight changes in the relative abundances of certain bacterial groups were recorded. For example, an uncultured *Acidobacterium* was significantly affected by herbivory. As part of the lysimeter experiment, we also investigated the influence of sward composition and the different management regimes on the bacterial communities in the rhizosphere. Both the herbicide application and lower mowing frequencies decreased the bacterial richness in the rhizosphere. Moreover, no differences in bacterial richness between fertilized and unfertilized plots were recorded. Further analyses revealed that a variety of distinct bacterial groups and species in the rhizosphere do respond to the treatments studied. For example, the abundance of the *Acidobacteria* was significantly reduced in fertilized plots. The opposite was observed for the *Actinobacteria*.

In conclusion, plant-associated bacteria in the endosphere and in the rhizosphere are affected by management regimes. Evaluating the impact of different grassland management regimes and above-ground herbivory onto plant-associated bacteria may results in a better understanding of the multitrophic interaction between plant species, bacterial communities, and above-ground herbivores. Furthermore, the results of this study will help to predict the impact of different grassland management regimes onto plant-associated bacterial communities and related effects on soil ecosystems.

2 ZUSAMMENFASSUNG

In den vergangenen Jahren rückten Pflanzen-assoziierte Bakterien auf Grund ihrer Bedeutung für die Pflanzengesundheit und das ökologische Gleichgewicht zunehmend in den Fokus aktueller Forschungen. Trotz der stetig steigenden Zahl wissenschaftlicher Studien ist der Einfluss von Bewirtschaftungsmaßnahmen auf die Diversität dieser Bakteriengemeinschaften in Grünlandökosystemen vergleichsweise wenig untersucht. In dieser Studie haben wir neue und interessante Erkenntnisse über die Diversität von Pflanzen-assoziierten Bakterien in Grünlandökosystemen gewonnen.

Sämtliche Untersuchungen dieser Arbeit wurden auf der GrassMan-Fläche in den Mittelgebirgslagen des Solling in Deutschland durchgeführt. Das GrassMan-Experiment wurde 2008 in einer Matrix von Wiesenplots schachbrettartig auf historisch altem Grünland errichtet. Die Bewirtschaftungsintensität unterschied sich bezüglich der Häufigkeiten (einmal jährlich im Juli oder dreimal jährlich im Mai, Juli und September) und der Düngung (keine Düngung bzw. Düngung mit NPK). Außerdem wurde durch gezielten Herbizid-Einsatz gegen Monokotylen oder gegen Dikotylen ein Gradient in der Anzahl der Pflanzenarten erzeugt.

Die Arbeit umfasst drei Hauptthemen. Erstens wurde der Einfluss verschiedener Bewirtschaftungsmaßnahmen auf die bakterielle Endophytengemeinschaft in den drei Grasarten *Festuca rubra*, *Lolium perenne* und *Dactylis glomerata* untersucht. Hierfür wurden im September 2010 und im April, Juli und September 2011 Pflanzenproben auf den Dikotylen-reduzierten Plots gesammelt. Die Umwelt-DNS wurde aus den Proben extrahiert und als Template für 16S PCRs eingesetzt. Die Struktur der bakteriellen Endophyten-Gemeinschaft wurde mittels DGGE-Analyse der erhaltenen PCR-Produkte untersucht.

Wir konnten Unterschiede der Endophyten-Gemeinschaftsstrukturen hinsichtlich der verschiedenen Bewirtschaftungsintensitäten feststellen. Während die Düngung einen starken Effekt auf die bakterielle endophytische Diversität sowohl in *F. rubra* als auch in *L. perenne* hatte, wurden die bakteriellen Endophyten in *D. glomerata* nicht dadurch beeinflusst. Die Proben von *L. perenne*, die von den ungedüngten Plots stammten, bildeten zudem eindeutige Gruppen bei der

Analyse der DGGE-Banden bezüglich der zwei Schnitthäufigkeiten. Somit beeinflusste auch die Mahd die bakterielle Endophyten-Gemeinschaft in den Pflanzen. Weiterhin konnten wir einen starken saisonalen Effekt auf die Struktur der endophytischen Gemeinschaft nachweisen. Da saisonale Veränderungen und die Pflanzenart die Zusammensetzung der endophytischen Bakteriengemeinschaft beeinflussten, können sich die Auswirkungen unterschiedlicher Bewirtschaftungsintensitäten mit der Zeit und der untersuchten Pflanzenart verändern. Dieses Ergebnis sollte bei zukünftigen Studien berücksichtigt werden.

Das zweite Ziel dieser Arbeit war die Beantwortung der Frage, wie sich oberirdische Herbivorie auf die Bakteriengemeinschaft in der Rhizosphäre auswirkt. Hierfür wurde im Herbst 2010 ein Lysimeter-Experiment auf der GrassMan-Fläche errichtet. Nach einer zweiwöchigen Herbivorie durch Grashüpfer und Schnecken im Sommer 2011 wurden Bodenproben von jedem Lysimeter genommen. Um Einblicke in die Zusammensetzung der bakteriellen Gemeinschaft in der Rhizosphäre zu erhalten, wurde die Gesamt-DNS aus den Bodenproben extrahiert und als Template in 16S rDNS PCRs eingesetzt. Die Gemeinschaftsstruktur wurde mittels DGGE-Analyse bzw. Pyrosequenzierung der erhaltenen PCR Produkte untersucht. Die Herbivorie hatte keinen Einfluss auf die Anzahl der Bakterien (richness), während leichte Änderungen in der relativen Abundanz von einigen Bakteriengruppen festgestellt wurden. So war zum Beispiel die relative Abundanz einer unkultivierten *Acidobacterium*-Art in den Herbivorie-Lysimetern erhöht.

Bestandteil des Lysimeter-Experiments war zudem die Untersuchung des Einflusses der Pflanzenartenanzahl und der verschiedenen Bewirtschaftungsmaßnahmen auf die bakterielle Gemeinschaft in der Rhizosphäre. Der Einsatz von Herbiziden und eine niedrigere Schnittfrequenz reduzierten die Artenanzahl (richness) der Bakterien in der Rhizosphäre. Die Düngung hatte keinen Einfluss auf die Anzahl der Arten. Weitere Analysen zeigten, dass eine Vielzahl von verschiedenen bakteriellen Taxa in der Rhizosphäre durch die untersuchten Maßnahmen beeinflusst wurde. So war die Abundanz der *Acidobacteria* in den gedüngten Plots signifikant geringer. Das Gegenteil trat bei den *Actinobacteria* auf.

Abschließend lässt sich sagen, dass Pflanzen-assoziierte Bakterien sowohl in der Endosphäre und Rhizosphäre durch Bewirtschaftungsmaßnahmen beeinflusst werden. Die Untersuchung der Wirkung von verschiedenen Bewirtschaftungsintensitäten im Grünland und von oberirdischer Herbivorie auf Pflanzen-assoziierte Bakterien kann zu einem besseren Verständnis der multitrophischen Interaktionen zwischen Pflanzenart, Bakteriengemeinschaft und oberirdischen Herbivoren führen. Außerdem können uns die Ergebnisse dieser Arbeit helfen, die Effekte unterschiedlicher Bewirtschaftungsmaßnahmen auf Pflanzen-assoziierte Bakteriengemeinschaften und damit zusammenhängende Effekte auf das Bodenökosystem vorherzusagen.

CHAPTER B

GENERAL INTRODUCTION

1 GRASSLANDS

Grasslands are found in every region of the world. They cover 3.5 billion hectare (ha) of the terrestrial surface area (Carlier *et al*., 2009). In Europe, grasslands are one of the most important land use forms (Isselstein *et al*., 2005). Around 160 million ha of the EU-27 were utilized for agriculture in 2007. This represents over one third of the entire EU territory. More than 33% of the total agricultural area utilized in Europe is covered by permanent grassland (EUROSTAT).

There is an increasing interest in grasslands due to the wide range of functions and utilizations with regard to the landscape and the environment (Gibon, 2005; Isselstein *et al.*, 2005; Carlier *et al*., 2009). This ecosystem plays an important role in agriculture and biodiversity conservation. For example, grasslands act as carbon sinks, water regimes regulators, erosion preventives, and as nitrogen fixation sources (Carlier *et al*., 2009). Moreover, they offer ideal habitats for a wide range of microorganisms, animal and plant species, as well as breeding grounds for many invertebrate and vertebrate species (Plantureux *et al*., 2005; Carlier *et al*., 2009).

Since the World War II, grasslands have undergone important changes. Different management regimes have been applied to increase primary production (Carlier *et al*., 2009). These regimes include, for example, the application of pesticides and chemical fertilizer, frequent mowing, and livestock grazing. This land use intensification of agricultural ecosystems causes many negative environmental effects, such as soil degradation, pesticide and fertilizer leaching (Stoate *et al*., 2001), the development of pesticide-tolerant bacteria (Shafiani & Malik 2003) and the loss of biodiversity (Isselstein *et al*., 2005; Tscharntke *et al*., 2005). Furthermore, it has been shown that changes in the soil environment (Stoate *et al*., 2001; Plantureux *et al*., 2005) as well as in the soil microbial community composition (Steenwerth *et al*., 2002; Li *et al*., 2012) were associated with different management regimes in grasslands. However, the influence of these regimes on plant-associated bacterial communities still remains largely unknown.

2 PLANT-ASSOCIATED BACTERIAL COMMUNITIES

2.1 PLANTS AS HABITATS FOR BACTERIA

Terrestrial plants offer diverse habitats for bacterial microorganisms by providing various nutrients, an environment protected from most biotic and abiotic parameters as well as physical structures for protection and attachment (Kowalchuk *et al.*, 2010). Plant-associated bacterial communities are able to colonize the above- and below-ground plant surfaces (phyllosphere and rhizosphere, respectively) as well as the endosphere (Fig. 1).

Fig. 1. General overview of plant-associated bacterial communities. These bacteria are endophytic (living inside tissues of healthy plants), epiphytic (colonizer of above-ground plant surfaces), and rhizospheric (colonizing the rhizosphere or the root surface). ○ = epiphytes, ◇ = rhizobacteria, ▲ = endophytes.

Depending on the chemical, biological, and physical characteristics of these three main habitats, plants offer distinct niches which require specifically adapted microorganisms. As a consequence, a wide range of microbial species is supported. In addition to bacteria, plants may be colonized by fungi, archaea, protista, oomycota, and nematodes. In this study, we specifically focused on plant-associated bacteria in the endosphere and in the rhizosphere.

2.1.1 THE RHIZOSPHERE

The active soil layer surrounding the roots and being influenced by living roots is defined as the plants rhizosphere (Sørensen, 1997). Compared to most soils, the rhizosphere is nutrient rich (Beattie, 2006). Bacteria living in this habitat have different types of metabolism pathways and adaptive responses to the supply of various nutrients, water, organic carbon sources, and oxygen (Sørensen, 1997; Beattie, 2006). For example, they are able to form close mutualistic relationships with plants and benefit from nutrients provided by root exudates. Consequently, the biomass and activity of bacteria in the rhizosphere is significantly higher compared to the bacterial biomass in the surrounding bulk soil (Sørensen, 1997; Raaijmakers *et al*., 2009). This effect is called the rhizosphere effect (Katznelson *et al*., 1948).

2.1.2 THE ENDOSPHERE

There are many different definitions for endophytes depending on the researchers' perspective. Taken literally, the term endophyte means "in the plant" (endon Greek, within; phyton: plant). According to Hallmann *et al*. (1997), endophytic bacteria are defined as those bacteria that can be extracted from within plants or isolated from surface-disinfected plant tissues, and that have no visibly harmful effects on the host plant.

Endophytes are found in a wide range of plants (Rosenblueth & Martinez-Romero, 2006) including grass species (Zinniel *et al*., 2002; Sun *et al*., 2008). Each individual plant is host to one or more endophytic species (Strobel & Daisy, 2003). Moreover, all plant parts such as fruits, seeds, leaves, stems, tubers, and

roots, respectively, are colonized by these bacteria (Hallmann *et al*., 1997; Sturz *et al*., 1997). Generally, endophytic bacteria have lower population densities than rhizospheric bacteria (Hallmann *et al*., 1997; Rosenblueth & Martinez-Romero, 2004). Some authors assume that endophytic bacteria are considered to be a subset of the bacteria community in soil or rhizosphere (Seghers *et al*., 2004; Gottel *et al*., 2011). Some of the bacteria in the rhizosphere or soil have developed mechanisms to penetrate and colonize plant tissues (Quadt-Hallmann *et al*., 1997; Reinhold-Hurek & Hurek 1998). Plant wounding either by abiotic (e.g., tillage, extreme temperature fluctuations) or by biotic factors (fungi, plant-parasitic nematodes, insects) may also result in endophytes entering plant tissue (Siddiqui & Shaukat, 2003).

2.2 IMPORTANCE OF PLANT-ASSOCIATED BACTERIA

Recently, plant-associated bacterial communities and their functions in grasslands have been investigated in more detail. This interest was fueled by studies showing bacteria to be able to produce biologically active metabolites such as antibiotic and antiparasitic agents with beneficial effects on associated plants (Kloepper *et al*., 1999; Compant *et al*., 2005). Despite their importance for agriculture, more research is needed to characterize the composition and activity of plant-associated bacteria and to analyze the interactions between plants and their associated bacterial communities.

Plants benefit from endophytic and rhizospheric bacteria in many ways. Bacteria in the rhizosphere and in the endosphere promote biological nitrogen fixation (Stoltzfus *et al*., 1997; Reinhold-Hurek & Hurek, 1998) as well as plant growth and health (Compant *et al*., 2010). They may cause a higher resistance to plant pathogens (Kloepper *et al*., 1992; Araujo *et al*., 2002) and parasites such as nematodes (Kloepper *et al*., 1992, Hallmann *et al*., 1998; Siddiqui & Shaukat, 2003). Moreover, they improve plant fitness towards environmental stresses (Sturz & Nowak 2000; Compant *et al*., 2010). Thus, endophytic as well as rhizospheric bacteria play an important role in agriculture and in the maintenance of environmental balance.

2.3 EFFECT OF ABIOTIC AND BIOTIC CHANGES ON PLANT-ASSOCIATED BACTERIAL COMMUNITIES

Plant-associated habitats are a dynamic environment. The diversity, activity, and species composition of bacterial communities in these habitats is affected by several abiotic and biotic factors such as plant species, crop rotation, or soil conditions (Hallmann *et al.*, 1997; Sørensen, 1997; Smalla *et al.*, 2001; Kent & Triplett, 2002; Kuklinsky-Sobral *et al.*, 2004). Moreover, grassland management regimes including fertilizer application influence the bacterial community in soil and rhizosphere (Clegg *et al.*, 2003; Doi *et al.*, 2011). As mentioned earlier, endophytic bacteria are considered to be a subset of the bacteria community in soil or rhizosphere (Seghers *et al.*, 2004; Gottel *et al.*, 2011). Consequently, management regimes influencing the community composition of bacteria in the rhizosphere might also affect bacterial community structures in the endosphere.

2.4 INVESTIGATION METHODS

Recently published studies concerning rhizospheric and endophytic bacteria and their community structures have been mainly based on culture-dependent methods**.** Most microorganisms (> 99%), however, cannot be cultivated using standard laboratories techniques (Amann *et al.*, 1995). Thus, the majority of plant-associated microbes have not yet been cultured in the laboratory (Araujo *et al.*, 2002; Kent &Triplett, 2002).

To overcome the limitations of culture-dependence, several culture-independent molecular approaches have been developed. The use of these approaches has provided substantial insight into our understanding of diversity, ecology, and physiology of microbial communities. For example, denaturing gradient gel electrophoresis (DGGE) or next generation sequencing of environmental 16S rRNA genes have been successfully applied to investigate bacterial communities in a great variety of environments including endosphere (Garbeva *et al.*, 2001; Araujo *et al.*, 2002; Hardoim *et al.*, 2012) as well as rhizosphere and soil (Smalla *et al.*, 2001; Nacke *et al.*, 2011).

3 EXPERIMENTAL SITE

The Grassland Management Experiment (GrassMan) has been established as a long-term field experiment with different management intensity treatments. In spring 2008, it was set up at a semi-natural, moderately species-rich grassland site at the experimental farm Relliehausen in the Solling Mountains in Lower Saxony, central Germany (51°44'53" N, 9°32'43" E, 490 m a. s. l.). In this region, the mean annual temperature is 6.9°C and the mean annual precipitation is 1028 mm (Deutscher Wetterdienst 1960 – 1990, station Silberborn-Holzminden, 440 m a.s.l.).

This permanent grassland site has been traditionally used as an extensive pasture and meadow since the end of the 19th century (Petersen *et al.*, 2012). It is a slightly sloping (ca. 5°) grassland area of 4 ha size. According to Petersen *et al.* (2012), the number of plant species ranged from 13 to 17 in 9 m^2 phytosociological relevés. The vegetation consists of a nutrient poor, moderately wet Lolio-Cynosuretum with high abundances of *Agrostis capillaris* L. and *Festuca rubra* L. (Petersen *et al.*, 2012). The dominating soil type of the experimental area has been determined as a shallow (40–60 cm), stony Haplic Cambisol (Keuter *et al.*, 2013) with a pH_{KCl} ranging from 4.18 to 5.47.

The full-factorial design of GrassMan includes two mowing frequencies (once per year in July vs. three cuttings in May, July, and September) and two fertilization treatments (no vs. NPK fertilization). The N fertilizer was applied as calcium ammonium nitrate N27 in two equal doses (180 kg N ha^{-1} yr^{-1}) in April and end of May. Additionally, 30 kg P ha^{-1} yr^{-1} plus 105 kg K ha^{-1} yr^{-1} as Thomaskali® (8% P_2O_5, 15% K_2O, 20% CaO) were applied at the end of May. These scheduled mowing and fertilization regimes started in 2009. Cuttings of plots to a height of 7 cm were done using a Haldrup® harvester.

A third factor aimed at varying plant diversity in the GrassMan plots. The three sward compositions (monocot-reduced, dicot-reduced, species-rich as control) were manipulated by selective herbicide applications targeting either dicots or monocots. To decrease the amount of monocots or dicots, a third of the plots was treated with either the herbicide Select 240 EC® (Stähler Int., Stade, Germany; active ingredients: Clethodim (0.5 l ha^{-1}) or with the herbicide mixture

Starane® and Duplosan KV (active ingredients: Mecoprop-P® and Fluroxypyr/ Triclopyr; 3 l ha^{-1} each), respectively. The application of herbicides took place on 31st of July 2008 resulting in significant changes in species richness and in functional group abundances (Petersen *et al.*, 2012). One third of the plots remained untreated and was used as controls (species-rich).

Each treatment was replicated six times resulting in 72 plots of 15 x 15 m size. The experimental layout was a Latin rectangle design, arranged in 6 rows and 12 columns, two columns forming one block (Fig. 2). The distance between rows and columns was 5 m and the distance within columns 3 m.

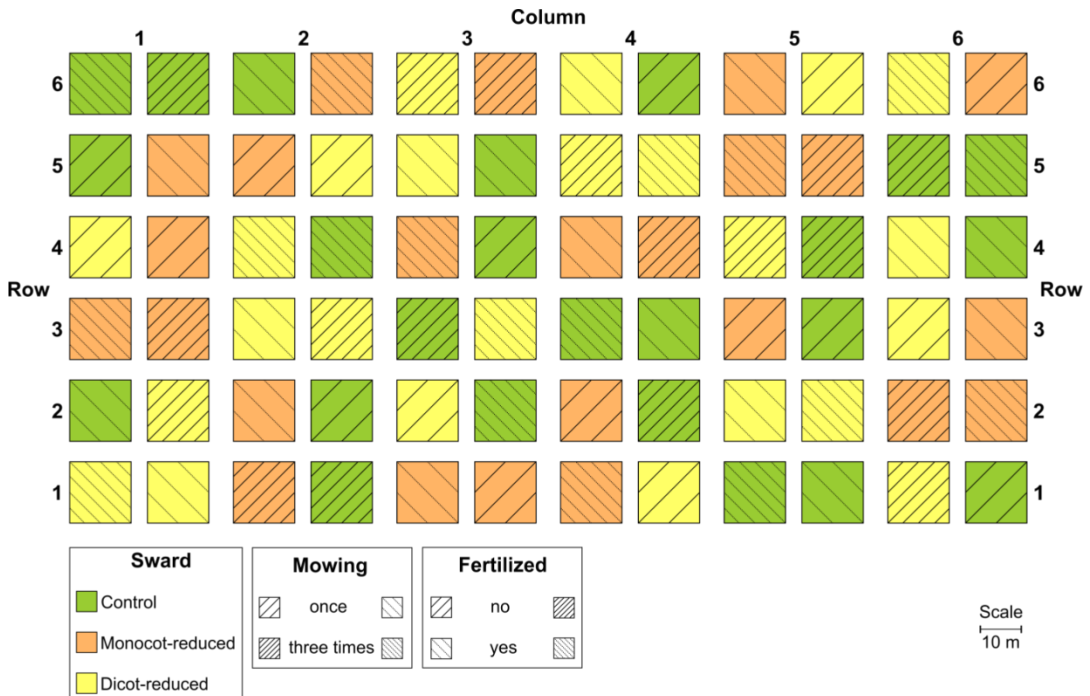

Fig. 2. Experimental design of the GrassMan experimental field in the Solling Mountains in Lower Saxony, central Germany (51°44'53" N, 9°32'43" E, 490 m a. s. l.). The full-factorial design of this study included two mowing frequencies (mown once per year in July vs. three cuttings in May, July, and September), two fertilization treatments (no vs. NPK fertilization), and three different plant diversity levels (monocot-reduced, dicot-reduced, species-rich as control).

4 GENERAL STUDY AIMS

Recently, plant-associated bacterial communities attracted the attention of research groups due to their importance for plant health and the environment. Despite the increasing number of papers on plant associated bacterial communities, only a limited number of studies have been published on the influence of management regimes on the diversity of plant-associated bacteria in grassland ecosystems.

This thesis concentrates on the effects of different management regimes and above-ground herbivory on plant-associated bacteria in the plant rhizosphere and in the endosphere of three abundant grass species. These investigations were carried out in the same area, the GrassMan experimental field in the Solling Mountains, central Germany. The three major aims were:

1. To investigate the influence of different mowing and fertilization regimes on the bacterial endophytic diversity in the three grass species *Festuca rubra* L., *Dactylis glomerata* L., and *Lolium perenne* L. To answer this question, plant samples were collected in September 2010 and 2011 from dicot-reduced plots. To further validate a seasonal effect on endophytic bacteria, samples were collected in April and July 2011 from three times mown, fertilized dicot-reduced plots.
2. To analyze the effects of different mowing and fertilizer regimes on the bacterial diversity in the rhizosphere. Thereto, samples were taken in summer 2011 and further studied with two different culture-independent approaches.
3. To investigate the impact of above-ground herbivory on the bacterial community in the rhizosphere. Following a two-week grasshopper and snail herbivory, soil samples were collected in summer 2011 and further analyzed employing two different metagenomic approaches.

CHAPTER C

PUBLICATIONS

STUDY 1

IMPACT OF GRASSLAND MANAGEMENT REGIMES ON BACTERIAL ENDOPHYTE DIVERSITY DIFFERS WITH GRASS SPECIES AND SEASON

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Performed the experiments: FW, DK

Analyzed data: FW, BW

Wrote the publication: FW, RD, SV

Conceived and designed the experiments: FW, SV

Abstract

Most plant species are colonized by a diverse number of microorganisms including endophytic bacteria. Despite their importance for plant health and yield, the response of these bacteria to grassland management regimes is still largely unexplored. This study aimed at assessing the bacterial endophytic community structure in the agricultural important grass species *Lolium perenne* L., *Dactylis glomerata* L., and *Festuca rubra* L. with regard to different fertilizer and mowing treatments. For that purpose, above-ground plant material from the Grassland Management Experiment (GrassMan) in Germany was collected in September 2010 and 2011. To evaluate seasonal effects, additional samples were taken in April and July 2011. DNA was extracted from the plant material and subjected to 16S rRNA gene PCRs. The endophytic community structure was subsequently studied by Denaturing Gradient Gel Electrophoresis (DGGE). Management regimes did not impact the endophytic community structure in the grasses in the same manner. Fertilization and mowing frequency significantly altered the endophytic communities in *L. perenne* and *F. rubra* but not in *D. glomerata*. On the other hand, season significantly affected the community structure in all three grass species. Moreover, as community structures were subjected to temporal variations, the recorded impact of management regimes differed between the two investigated years.

Introduction

Almost all plant species are colonized by a high number of microorganisms including endophytic bacteria (Senthilkumar *et al.*, 2011). Endophytic bacteria are defined as bacteria that can be extracted from within plants or isolated from surface-disinfested plant tissue, and that have no visibly harmful effects on the plant (Hallmann *et al.*, 1997). They are found in a wide range of plants (Sturz *et al.*, 2000).

Many biotic factors including plant species, plant age, plant tissue, or the presence of phytopathogenic fungi, as well as abiotic factors such as soil conditions, temperature, or crop rotation influence the bacterial endophytic

community (e.g., Hallmann *et al.*, 1997; Fuentes-Ramírez *et al.*, 1999; Sessitsch *et al.*, 2002; Seghers *et al.*, 2004; Hardoim *et al.*, 2012). Moreover, plant species vary in their biochemical composition, which may affect the endophytic bacterial community (Hallmann & Berg, 2006). As endophytic bacteria rely on the nutritional supply offered by the plant, any factor influencing the nutritional or physiological status of the plant may consequently have an impact on the endophytic community (Hallmann *et al.*, 1997; Fuentes-Ramírez *et al.*, 1999).

Several endophytic bacteria have been reported to promote plant growth, plant yield, and the overall plant health by a number of mechanisms. These include the production of phytohormones and antibiotics (Bacon & Hinton, 2006; Compant *et al.*, 2010) as well as enhanced nutrient availability and nitrogen fixation (Stoltzfus *et al.*, 1997; Rosenblueth & Martinez-Romero, 2006). Furthermore, plants infected with endophytic bacteria have a higher resistance to plant pathogens (e.g., Hallmann *et al.*, 1998; Hallmann, 2001; Krechel *et al.*, 2002; Siddiqui & Shaukat, 2003; Compant *et al.*, 2005) and environmental stresses (Sturz & Nowak, 2000; Bacon & Hinton, 2006; Bacon & Hinton, 2011).

Although their important role in agricultural cropping systems is frequently appreciated (e.g., Hallmann *et al.*, 1997; Kobayashi & Palumbo, 2000; Bacon & Hinton, 2006; Maksimov *et al.*, 2011; Senthilkumar *et al.*, 2011), the diversity of interactions between endophytic bacteria, plant species, and management regimes is not fully understood. Previous studies on the impact of different management regimes, such as fertilizer application, have mainly focused on root endophytic bacteria (Tan *et al.*, 2003; Seghers *et al.*, 2004; Kuklinsky-Sobral *et al.*, 2005), and nitrogen-fixing (diazotrophic) bacteria (Fuentes-Ramírez *et al.*, 1999; Sturz *et al.*, 2000; Tan *et al.*, 2003; Doty *et al.*, 2009; Prakamhang *et al.*, 2009).

The aim of this study was to investigate the influence of combined fertilizer applications and mowing regimes as well as the effect of season on the overall diversity of bacterial endophytes in three abundant and important agricultural grass species (*Dactylis glomerata* L., *Festuca rubra* L., and *Lolium perenne* L.). We hypothesized (1) that the overall endophytic community structure is different between the three examined grass species as the grasses differ in their physiological state. We further hypothesized (2) that the overall bacterial

endophytic community structure of the investigated grasses is influenced by fertilizer application and different mowing frequencies as these management regimes affect the host plants and, thus, indirectly the endophytes in the grasses. Moreover, we hypothesized (3) that the endophytic community in the grass species is influenced by season as the physiological state of the plant is altered with season.

For this purpose, above-ground plant material was taken from the Grassland Management Experiment (GrassMan), a long-term experimental field on a semi-natural, moderately species-rich grassland site. The aim of this experiment was to investigate the effects of fertilizer application, mowing frequencies, and sward composition on diversity and ecosystem functioning. For this purpose, ten samples per grass species and plot were collected in both September 2010 and 2011. To investigate the influence of season on the endophytic communities, 10 samples per grass species were collected from fertilized plots in April and July 2011. DNA was extracted from the plant material and subjected to 16S RNA gene PCR. Obtained PCR products were subsequently studied by DGGE analysis. In addition to the culture-independent approach, non-specialized endophytes were isolated from the grass species and classified by 16S rRNA gene analysis.

Materials and Methods

Study site

The Grassland Management Experiment (GrassMan) is a long-term field experiment with different management intensity treatments. It was established in spring 2008 at a semi-natural, moderately species-rich grassland site in the Solling Mountains in Lower Saxony, central Germany (51°44'53" N, 9°32'43" E, 490 m a.s.l.). At least since the late 19th century, this grassland site has been traditionally used as pasture or for hay making (Geological Map of Prussia 1910 (based on the topographic inventory of 1896), topographic maps of Sievershausen and Neuhaus/Solling 1924, 1956 and 1974). The pasture has been improved by annual fertilization (80 kg N ha^{-1} yr^{-1}), liming, and overseeding with high value forage species (farm records Relliehausen since 1966). The moderate fertilization

stopped two years before the first experiments started. The vegetation consists of a nutrient poor, moderately wet Lolio-Cynosuretum (Petersen *et al.*, 2012). The mean annual temperature is 6.9°C and the mean annual precipitation is 1028 mm (Deutscher Wetterdienst 1960-1990, Station Silberborn-Holzminden, 440 m a.s.l.). During the study period, mean temperature and precipitation were 11.42°C and 93.6 mm in September 2010, 11.26°C and 41.75 mm in April 2011, 14.48°C and 110.85 mm in July 2011, and 14.75°C and 54.75 mm in September 2011, respectively. The dominating soil type of the experimental area has been determined as a shallow (40-60 cm), stony Haplic Cambisol (Keuter *et al.*, 2013) with a pH_{KCl} ranging from 4.18 to 5.47.

Experimental design

The three-factorial design of this study included two mowing frequencies (once per year in July vs. three times per year in May, July, and September) and two fertilizer treatments (no vs. NPK fertilizer application). All plots were cut to a height of 7 cm with a Haldrup® harvester. The N fertilizer was applied as calcium ammonium nitrate N27 in two equal doses (180 kg N ha^{-1} yr^{-1}) in April and end of May. In addition, 30 kg P ha^{-1} yr^{-1} plus 105 kg K ha^{-1} yr^{-1} as Thomaskali® (8% P_2O_5, 15% K_2O, 20% CaO) were also applied at the end of May. A third parameter manipulated was the sward composition (monocot-reduced, dicot-reduced, species-rich). This was achieved by selective herbicide application which either reduced dicot (Mecoprop-P and Fluroxypyr/ Triclopyr; 3 l ha^{-1} each) or monocot species diversity (Clethodim; 0.5 l ha^{-1}). One third of the plots was left untreated as control (species-rich). The application of herbicides took place on 31st July 2008 resulting in significant changes in species richness and in functional group abundances (Petersen *et al.*, 2012). Each treatment was replicated six times, resulting in 72 plots of 15 x 15 m size arranged in a Latin rectangle.

Sampling

Above-ground plant material was collected on 19th September 2010 and on 12th September 2011 (shortly before the third annual mowing application) from dicot-reduced plots. To investigate seasonal effects on the bacterial endophytic community structure in the three investigated grass species, samples from the

intensively managed (fertilized, thrice mown), dicot-reduced plots were additionally collected on 12th April 2011 (prior to fertilizer application or mowing) and on 18th July 2011 (after fertilizer application and shortly before the second annual mowing application). Ten plants per grass species and plot were randomly selected for sampling, with one exception: due to the low number of *L. perenne* in the plots mown once a year in September 2010, above-ground plant material was collected only from two non-fertilized and from three fertilized plots.

Collected plants did not show obvious disease symptoms, such as leaf spots, chlorosis, or other types of pathogen-induced lesions. Following cutting of above-ground plant material with sterilized scissors, the collected plant samples were immediately cooled down (below 4°C) and transported to the laboratory. Plant material derived from the same plot and plant species was pooled prior to surface sterilization.

Surface sterilization of plants

Surface-sterilization of plant tissues was performed according to Schulz *et al.* (1993), with slight modifications. Plant material was immersed in 37% formaldehyde for 3 min and rinsed two times with autoclaved and sterile-filtered water. To remove DNA, samples were rinsed with DNA-Exitus (Applichem, Darmstadt, Germany) for 30 s and subsequently washed three times in autoclaved and sterile-filtered distilled water. To control the success of the applied surface sterilization, water from the third wash step was plated on common laboratory media plates, i.e., malt extract agar (MEA), Luria-Bertani-Agar (LB), and potato dextrose agar (PDA). The plates were incubated in the dark at 25°C for at least two weeks. No growth of microorganisms was observed. The surface-sterilized plant material was triturated with an autoclaved mortar and pestle. The powdered samples were stored at -80°C until DNA extraction.

Isolation of non-specialized endophytes

For the isolation experiment, surface-sterilized plant material from 9 plots (at least 2 of each treatment) was cut into several pieces of approximately 5 to 15 mm length. Ten to 15 plant fragments were placed on malt extract agar (MEA), Luria-Bertani-Agar (LB), and potato dextrose agar (PDA) plates. Moreover, at least 10

plant fragments were incubated in 1 mL NaCl-solution (1% (w/v). The tubes were extensively shaken for 10 s and then incubated for 20 to 30 min. Prior to shaking, five to six glass beads (3 mm) were added to increase the extraction efficiency. 400 µl of the resulting solution were pipetted onto an agar plate. The plates were incubated in the dark at 25°C for at least two weeks. Colonies were further cultivated in liquid culture (LB media). After one day growing at 25°C, DNA was extracted using the peqGold Plant DNA Mini Kit (Peqlab, Erlangen, Germany) were subjected to PCR-based amplification targeting the bacterial 16S rRNA gene.

Amplification of the 16S rRNA genes of isolated endophytic strains

PCR amplification of bacterial 16S rRNA genes was performed with the primers 8F 5'-AGAGTTTGATCMTGGC-3 (Muyzer *et al.*, 1995) and 1114R 5'-GGGTTGCGCTCGTTRC-3' (Wilmotte *et al.*, 1993). The PCR reaction mixture (25 µl) contained 2.5 µl of 10-fold Mg-free Taq polymerase buffer (Fermentas), 200 µM of each of the four desoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.4 µM of each primer, 5% DMSO, 0.5 U of *Taq* DNA polymerase (Fermentas), and approximately 10 ng of the DNA sample as template. Negative controls were performed by using the reaction mixture without template. The following thermal cycling scheme was used: initial denaturation at 95°C for 2 min and 25 cycles of: 1 min at 95°C, 1 min at 55°C and 1.5 min at 72°C. The final extension was carried out at 72°C for 5 min. The resulting PCR products were checked for appropriate size and then purified using the peqGOLD Gel Extraction Kit (Peqlab) as recommended by the manufacturer. Sequences of the purified PCR products were determined by Sanger sequencing at the Göttingen Genomics Laboratory.

Extraction of total community DNA

Total microbial community DNA was extracted employing the peqGOLD Plant DNA Mini Kit (Peqlab) according to the manufacturer's instructions with two modifications. Glass beads were used in the first step to grind plant material. Furthermore, 10 µl Proteinase K (20 mg mL^{-1}) were added to improve initial cell lysis. DNA was eluted in 30 µl Diethylpyrocarbonate (DEPC) water.

Amplification of the 16S rRNA genes for DGGE analysis

For DGGE analysis, a nested PCR approach was applied. In the first PCR, the primers 799f (AACMGGATTAGATACCCKG) and 1492R (GCYTACCTTGTTACGACTT) were used to suppress co-amplification of plant chloroplast 16S rRNA gene DNA (Chelius & Triplett, 2001). PCR amplification with this primer pair resulted in two PCR products: a mitochondrial product with approximately 1.1 kbp and a bacterial product of approximately 735 bp.

The PCR reaction mixture (25 µl) for amplification of the target gene contained 2.5 µl of 10-fold Mg-free *Taq* polymerase buffer (Fermentas, St. Leon-Rot, Germany), 200 µM of each of the four desoxynucleoside triphosphates, 1.75 mM $MgCl_2$, 0.4 µM of each primer, 5% DMSO, 1.5 U of *Taq* DNA polymerase (Fermentas), and approximately 25 ng of the DNA sample as template. Negative controls were performed by using the reaction mixture without template. Three independent PCR reactions were performed per sample and obtained PCR products were pooled in equal amounts. The following thermal cycling scheme was used: initial denaturation at 95°C for 5 min and thirty cycles of: 1 min at 94°C, 1 min at 53°C and 1 min at 72°C. The final extension was carried out at 72°C for 8 min. The resulting PCR amplicons were electrophoretically separated and bands specific for bacteria were excised from the gel. DNA was subsequently purified using the peqGOLD Gel Extraction Kit (Peqlab) according to manufacturer’s instructions.

Purified products were subjected to nested PCR with the primer pair F968-GC (5'- AACGCGAAGAACCTTAC-3') and R1401 (5'-CGGTGTGTACAAGACCC-3') (Nübel *et al.*, 1996). To prevent complete denaturation of the fragment, a GC-rich sequence (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') was attached at the 5'- end of the primer F968-GC (Muyzer *et al.*, 1993). The same PCR reaction mixture as for the first PCR was used for nested PCR with one modification: only 1 U of *Taq* DNA polymerase (Fermentas) was added to the mixture. The thermal cycling scheme of the nested PCR was as follows: initial denaturation at 94°C for 5 min, 11 cycles of: 1 min at 94°C, 1 min at 60°C (minus 1°C per cycle) and 2 min at 72°C, followed by 17 cycles of: 1 min at 94°C, 1 min at 53°C and 2 min at 72°C. The final extension was carried out at 72°C for

10 min. The resulting PCR products were checked for appropriate size by agarose gel electrophoresis. Three independent PCR reactions were performed per sample and obtained PCR products were pooled in equal amounts.

Denaturing Gradient Gel Electrophoresis (DGGE)

To investigate the bacterial endophytic diversity, the products derived from 16S rRNA gene PCRs were studied by DGGE analysis. DGGEs were carried out by using a PhorU2 machine (Ingeny, Goes, the Netherlands) with a double gradient. The first gradient ranged from 55 to 68% denaturant with a second gradient of 6.2 to 9% acrylamide. The acrylamide gradient was applied to enhance band sharpness and resolution (Cremonesi *et al.*, 1997). The denaturant (100%) contained 7 M urea and 40% formamide. Approximately 100 ng of the PCR product were loaded. The DGGE run was performed in 1xTris-acetate-EDTA buffer (40 mM Tris, 20 mM sodium acetate, 1 mM Na_2EDTA [pH 7.4]) at 60°C. Following electrophoresis for 16 h at 100 V, the gels were stained for 60 min with SYBRGold (Invitrogen, Darmstadt, Germany) and subsequently photographed on a UV transillumination table. To compare the reproducibility of the statistical analysis of the DGGE profiles, at least two independent DGGE runs were performed.

DGGE data analysis

Analysis of DGGE profiles was carried out using the software package GELCOMPAR II, version 5.1 (Applied Math, Ghent, Belgium). Cluster analyses (UPGMA) based on Jaccard correlation indices considering band presence and absence were performed to evaluate the percentage of similarity shared among the samples from the different treatments and sampling dates. Due to the low plant number obtained for *L. perenne* in September 2010, these data were excluded from the cluster analysis. To further evaluate the impact of management regimes and sampling time, the results of the DGGE were analysed in R employing the vegan package (version 3.0.1). For this purpose, similarity matrices exported from GelCompare were converted into dissimilarity objects and subsequently analysed by *Permutational Multivariate Analysis of Variance Using Distance Matrices* (*adonis*) [http://cran.r-project.org/web/packages/vegan/vegan.pdf].

Identification of abundant bacterial community members by DGGE

To identify the most abundant members of the bacterial endophytic community, several dominant bands were excised from DGGE gels, re-amplified, and sequenced. Excised bands were incubated in 30 µl sterile TE buffer (pH 8) overnight at 4°C. One µl of the resulting solution was subjected to PCR reaction to re-amplify the 16S rRNA gene fragment. The PCR was performed as described for the nested PCR reaction with one exception: the forward primer F968 did not carry the GC clamp. The resulting PCR products were checked for appropriate size and purified using the peqGOLD Gel Extraction Kit (Peqlab) as recommended by the manufacturer. The Göttingen Genomics Laboratory determined the sequences of the purified PCR products by Sanger sequencing.

Further Analysis of 16S rRNA gene sequences

All obtained 16S rRNA gene sequences were further analyzed employing the QIIME software package (version 1.6) (Caporaso *et al.*, 2010) and other tools. The Uchime algorithm implemented in Usearch (version 6.0.152) was initially applied in reference mode to identify and remove putative chimeric sequences using the most recent SILVA database (SSURef 115 NR) (Quast *et al.*, 2013) as reference dataset. Afterwards sequences were clustered into operational taxonomic units (OTUs) at 99% genetic similarity by BLAST alignment against the above-mentioned SILVA database using the pick_otus.py script (QIIME). The phylogenetic composition was determined by classifying the sequences with respect to the silva taxonomy of their closest match.

Nucleotide sequence accession numbers

Nucleotide sequences of the isolated strains and sequenced DGGE bands were deposited in GenBank under accession numbers KF699892 to KF699947 and KF699948 to KF700039, respectively.

Results and Discussion

Community structure differs with grass species and analysis approach

To assess endophytic community structures in the three grass species, DNA was extracted from plant material and subjected to 16S rRNA gene PCRs. Obtained PCR products were studied by DGGE analysis. DGGE fingerprints revealed patterns with 10 to 20 bands for each sample (Figs. S1-3). Prominent bands were excised and sequenced. Analysis of the obtained sequences revealed that bacterial diversity on class level was lowest and highest in *L. perenne* and *D. glomerata* (Fig. 1), respectively.

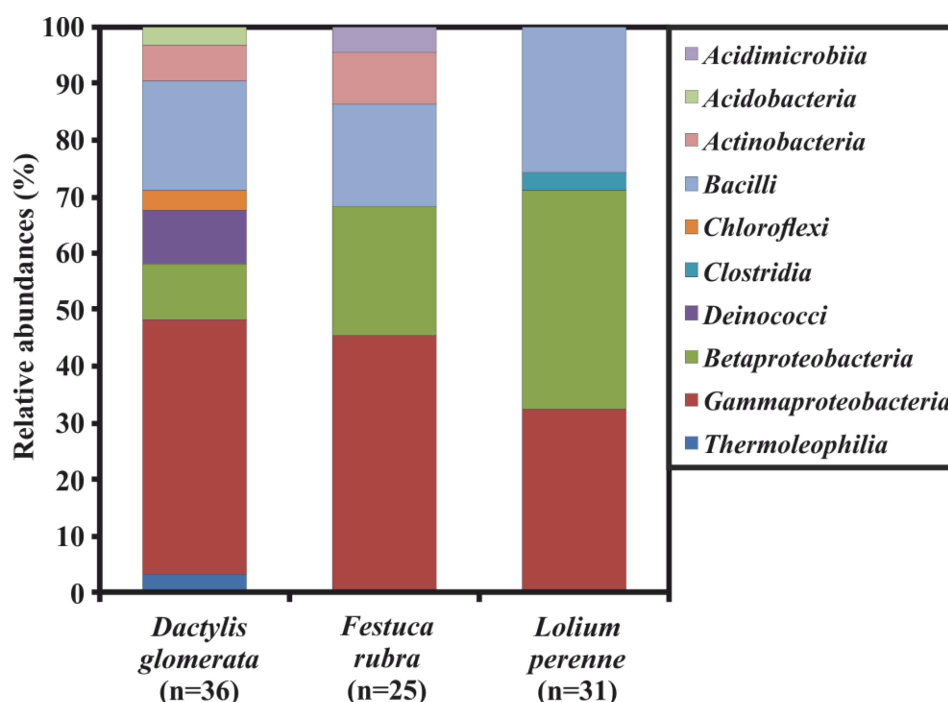

Fig. 1. Composition of the endophytic bacterial communities in the three grass species as revealed by sequencing of prominent DGGE bands. The number below the species name refers to the number of 16S rRNA genes sequences used in the analysis.

Gammaproteobacteria were the most dominant bacterial phylum in both *D. glomerata* and *F. rubra*. This is in agreement with other studies (Chelius & Triplett, 2001; Sun *et al.*, 2008; Gottel *et al.*, 2011). Endophytic bacteria in *L. perenne* were dominated by *Betaproteobacteria.* The second most dominant

groups were *Bacilli* (*D. glomerata*), *Betaproteobacteria* (*F. rubra*), or *Gammaproteobacteria* (*L. perenne*). Within the *Gammaproteobacteria*, we identified *Pseudomonas* as the most common genus (Table S1). One interesting species identified was *Herbaspirillum seropediacae* which is known as a nitrogen-fixing endophyte in sorghum, maize, sugarcane, and other plants (Baldani *et al.*, 1986; Olivares *et al.*, 1996).

We further examined how similar/dissimilar the endophytic communities are between the three investigated grass species. The number of calculated operational taxonomic units (OTUs) shared between the species was lower than the number exclusively found in one species (Fig. 2) which may refer to the different physiological states of the grass species investigated. Whereas 10 of the 29 identified OTUs of *D. glomerata* were also detected in *F. rubra* and *L. perenne*, the latter species shared 7 OTUs. Only 5 OTUs were found being present in all three grass species: one uncultured bacterium of the *Comamonadaceae*, *Staphylococcus aureus*, *S. epidermidis*, *Janthinobacterium lividum*, and *Pseudomonas balearica* (Table S1).

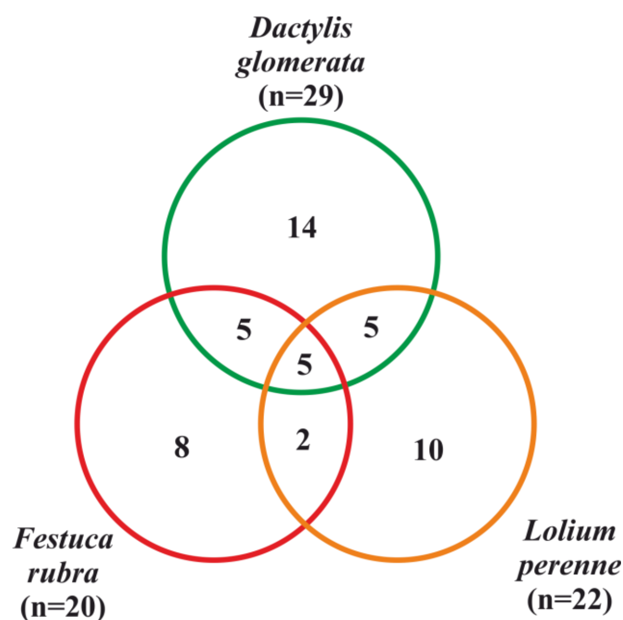

Fig. 2. Number of shared operational taxonomic units (OTUs) at 1% genetic distance. The number below the species name refers to the number of OTUs used in the analysis.

The recorded findings support our first hypothesis that the grass species differ in their endophyte community structure. This is in accordance with a study of McInroy and Kloepper (1995A) who found differences in the bacterial endophyte population in field-grown sweet corn and cotton grown side by side. They suggested that internal plant niches are colonized by a wide variety of bacteria. According to Hallmann (2001), the differences in bacterial endophytic community structures between different plant species growing next to each other can only be explained by plant species-specific selection mechanisms. Moreover, different plant species vary in their biochemical composition, which may affect bacterial endophyte community (Hallmann & Berg, 2006).

The spectrum of indigenous endophytic bacteria in roots is not only affected by niche specialization, but also by differences in colonization pathway (Hallmann & Berg, 2006). It is assumed that soil and rhizosphere are the main sources of endophytic colonizers (Hallmann & Berg, 2006). Many bacteria in these environments are able to penetrate and colonize root tissues (Quadt-Hallmann *et al.*, 1997; Reinhold-Hurek & Hurek, 1998). Plant wounding either by abiotic (e.g., tillage, extreme temperature fluctuations) or by biotic factors (e.g., fungi, plant-parasitic nematodes, insects) can also result in microbes entering the plant tissue (reviewed in Siddiqui & Shaukat, 2003). Other possible sources for endophytic bacteria include the anthosphere, the seeds, and the phyllosphere (Hallmann *et al.*, 1997; Hallmann, 2001; Compant *et al.*, 2010).

We also tried to assess the endophytic community structure by isolating strains from the three grass species. The most dominant groups isolated from the grasses were members of the *Bacilli* and *Gammaproteobacteria*, with *Pseudomonas* and *Bacillus* being the most abundant genera (Table S1). This is in accordance with other studies (as reviewed in Hallmann & Berg, 2006). However, a comparison of OTUs calculated for the 16S rRNA gene datasets obtained from the culturing-dependent and from the culturing-independent approach exhibited no overlap of the endophytic communities (Table S1). Consequently, the isolated strains do not necessarily represent the dominant endophytes in the three grasses.
This result is supported by other studies (e.g., Chelius & Triplett, 2001; Garbeva *et al.*, 2001; Araujo *et al.*, 2002; Conn & Franco, 2004). For example, *Araujo et al.* (2002) showed that some endophytic bacteria in citrus plants were only

observed by DGGE and not by the culture-dependent approach. In a study with potato plants, several non-culturable or so far uncultured endophytic organisms were detected. According to Chelius and Triplett (2001), the culturable component of the bacterial community reflected a community composition different from that of the clone library. Thus, only the community structures assessed by the metagenomic approach were further examined for their response to different management regimes and season.

Fertilizer application and mowing regimes differently shape bacterial endophytic community composition in D. glomerata, L. perenne, and F. rubra

In order to validate our second hypothesis that different fertilizer application and mowing regimes alter the bacterial endophytic communities, we compared DGGE band patterns with respect to the different management practises. UPGMA dendrograms of endophytic bacterial communities in *D. glomerata*, *L. perenne*, *and F. rubra* revealed differences with regard to fertilizer treatments and mowing frequencies (Figs. 3-5). Plants of *D. glomerata* sampled in September 2010 (Fig. 3A) and 2011 (Fig. 3B) did not cluster with respect to the applied management regimes. Furthermore, a significant influence of fertilizer application or mowing frequency was not recorded (Table 1). In contrast to *D. glomerata*, cluster analysis for *F. rubra* revealed a strong impact of the fertilizer treatment on bacterial endophytic community in September 2010 (Fig. 4A), but to a lesser extend in 2011 (Fig. 4B).

Furthermore, fertilizer application affected the community structure of bacterial endophytes in plants of *L. perenne* in September 2011 (Fig. 5). Such clear patterns were not recorded for the mowing regime. These results are in concordance with the statistical evaluation: fertilization and the interaction of fertilizer application and mowing frequency but not of mowing itself significantly influenced the structure of the endophytic community in *F. rubra* in September 2010 and in *L. perenne* in September 2011 (Table 1).

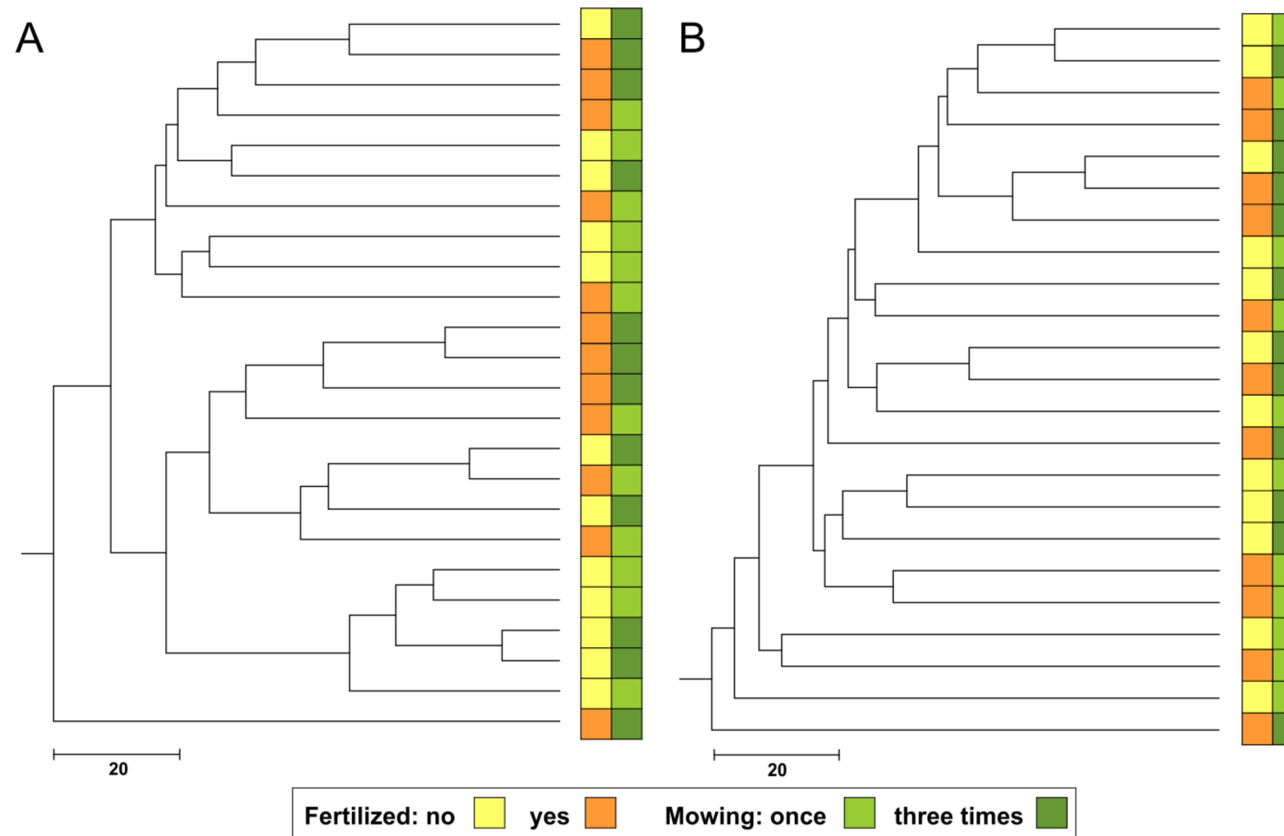

Fig. 3. UPGMA dendrogram generated by cluster analysis of DGGE fingerprints on the influence of different fertilization and mowing regimes on bacterial endophyte communities in above-ground plant parts of *D. glomerata*. Plant samples were taken in September 2010 (A) and 2011 (B). The dendrogram was constructed using the Jaccard correlation coefficient. The scale shows similarity values.

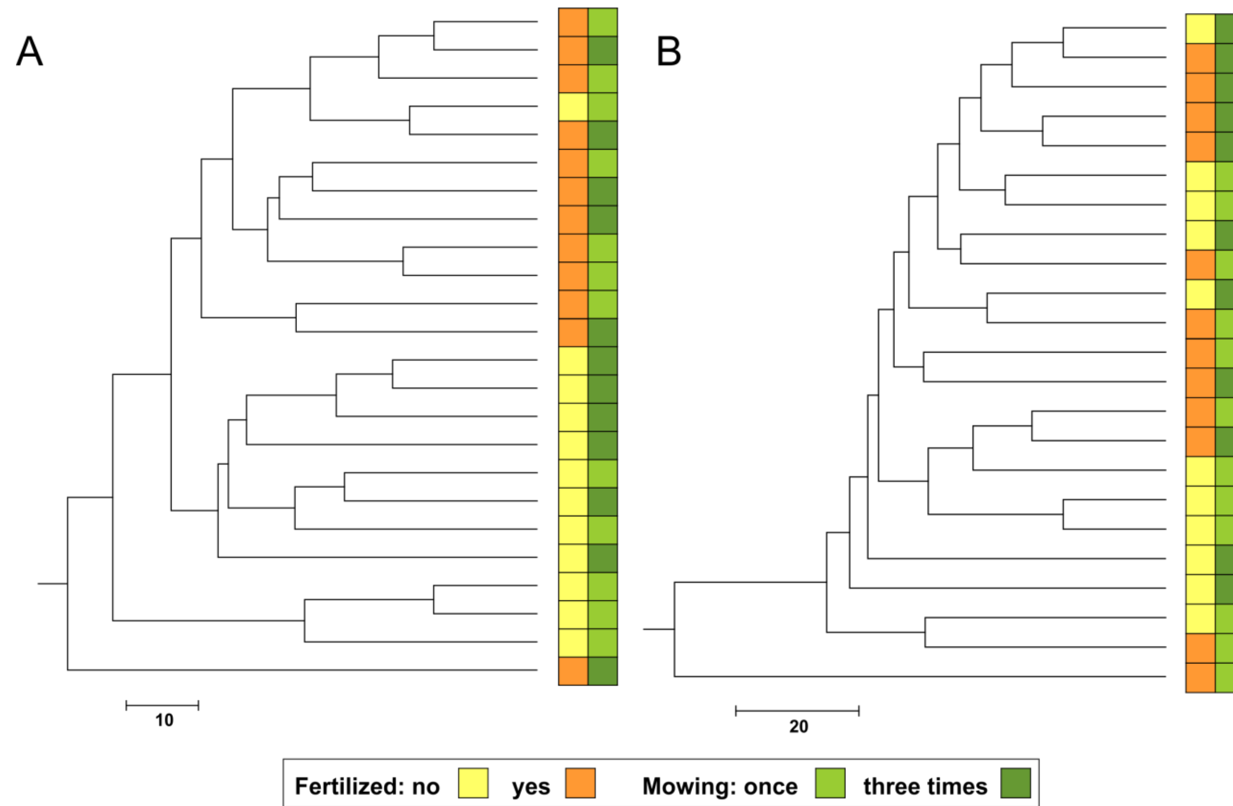

Fig. 4. UPGMA dendrogram generated by cluster analysis of DGGE fingerprints on the influence of different fertilization and mowing regimes on bacterial endophyte communities in above-ground plant parts of *F. rubra*. Plant samples were taken in September 2010 (A) and 2011 (B). For details see Fig. 3.

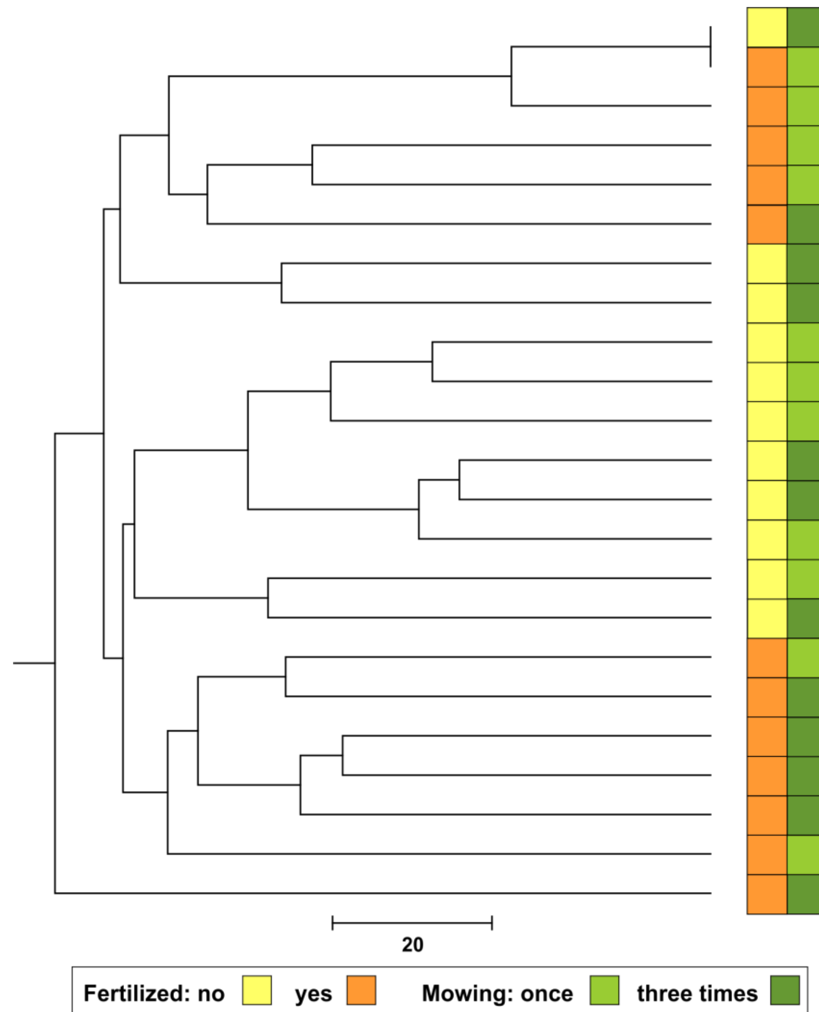

Fig. 5. UPGMA dendrogram generated by cluster analysis of DGGE fingerprints on the influence of different fertilization and mowing regimes on bacterial endophyte communities in above-ground plant parts of *L. perenne*. Plant samples were taken in September 2011. For details see Fig. 3.

Table 1. Statistical evaluation of the influence of management regimes and season towards the bacterial endophyte community in *D. glomerata*, *F. rubra*, and *L. perenne*. Abbreviation: Fert.:Mow. = the interaction of fertilization and mowing.

Species		*Management regimes*			*Time*	
		Fertilization	*Mowing*	*Fert.:Mow.*	*Season*	*Year*
D. glomerata	2010	-	-	-		
	2011	-	-	-		
	-				***	**
F. rubra	2010	**	-	***		
	2011	-	-	-		
	-				***	***
L. perenne	2010	NA	NA	NA		
	2011	*	-	**		
	-				***	***

not significant (-); significant with $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

It is well-known that different management practices have an impact on bacterial endophytic communities, but most previous research has focused on root endophytes (Hallmann *et al.*, 1999, Tan *et al.*, 2003; Seghers *et al.*, 2004; Kuklinsky-Sobral *et al.*, 2005) or on nitrogen-fixing (diazotrophic) endophytes (Fuentes-Ramírez *et al.*; 1999, Sturz *et al.*, 2000; Tan *et al.*, 2003; Doty *et al.*, 2009; Prakamhang *et al.*, 2009). For example, endophytic populations in cotton roots are affected by application of nitrogen-containing chitin as an organic amendment (Hallmann *et al.*, 1999). Moreover, a higher diazotrophic bacterial diversity in the roots of rice cultivated in unfertilized and previously uncultivated soil than in paddy soil amended with nitrogen fertilizer were recorded by Prakamhang *et al*. (2009). According to Tan *et al*. (2003), a rapid change of both the population and the activity of nitrogen-fixing bacteria in rice roots were observed within 15 days after N-fertilization.

Although these studies investigated the endophytic community in cotton and rice roots, they are in accordance with the results of the present study. Plant samples of *D. glomerata* in both years investigated and plant samples of *F. rubra* taken in September 2011 showed no significant impact of any management

regime. This result is concordant with a study of Seghers *et al.* (2004), which showed that mineral fertilizer as well as herbicide application exhibited no impact on bacterial endophytic community structure in maize kernels. The recorded findings partly support our initial hypothesis as some but not all investigated plant samples were affected by the applied management regimes.

Moreover, as the recorded effects on endophytic communities were different between the three grass species examined in this study, it is most likely that also the grasses are affected differently by management regimes which is in concordance with our second hypothesis.This was supported by an experiment in the Fraser Valley of British Columbia (Parish *et al.*, 1990). In five consecutive years, the authors investigated the effects of two different fertilizer levels (non-fertilized, fertilized) and four frequencies of mowing on the botanical composition of a pasture. At the end of the study, only *D. glomerata* was found in all treatments, while the abundance of *Lolium* spp. declined considerably. Mowing and fertilizer application every 3 weeks had a significant impact on the abundance of all investigated species. Furthermore, there was a significant fertilization - mowing interaction effect on all species except *Festuca* sp. The authors suggested that the plants differ in their growth rates and tolerance to shading and fertilizer application.

Additionally, the grass species investigated in this study differ in their indicator values such as tolerance against mowing or grazing (Dierschke & Briemle, 2002). Both *D. glomerata* and *L. perenne* have a higher tolerance against mowing compared to *F. rubra*. In contrast, *L. perenne* shows a higher indicator value for nitrogen than the other two grass species. As mentioned earlier, plants vary in their biochemical composition which might explain differences in the bacterial endophytic community (Hallmann & Berg, 2006). Hallmann *et al.* (1999) suggested that changes in plant physiology may result in the development of distinct bacterial endophytic communities. Moreover, endophytic bacteria rely on the nutritional supply offered by their host plant. As a consequence, changes in the nutritional or physiological status of the host plant may have an influence on the plant's endophytic community (Hallmann *et al.*, 1997; Fuentes-Ramírez *et al.*, 1999).

Seasonal impact on the abundance of bacterial endophytic community in the three grass species D. glomerata, F. rubra, and L. perenne

To verify our third hypothesis that the season has an effect on the bacterial endophytic community structure, we compared DGGE band patterns obtained from plant samples collected in September 2010 and April, July, and September 2011 (Figs. 6 and S4). Band patterns of *F. rubra* samples taken during the same season clustered together indicating a more similar community composition at the same season (Fig. 6B). Four of the six July samples cluster together with samples taken in September 2011. The other two samples showed higher similarities to samples taken in April 2011 and September 2010. This may indicate that the bacterial community composition in *F. rubra* followed a within year pattern. Plant species that propagate vegetatively are able to transmit their endophytes to the next generation so that no infection is required (Rosenblueth & Martinez-Romero, 2006). *Festuca rubra* is propagated mainly by rhizomes. Therefore, this propagation pattern might explain our findings that endophytic communities were quite similar in July and September 2011 in this grass species.

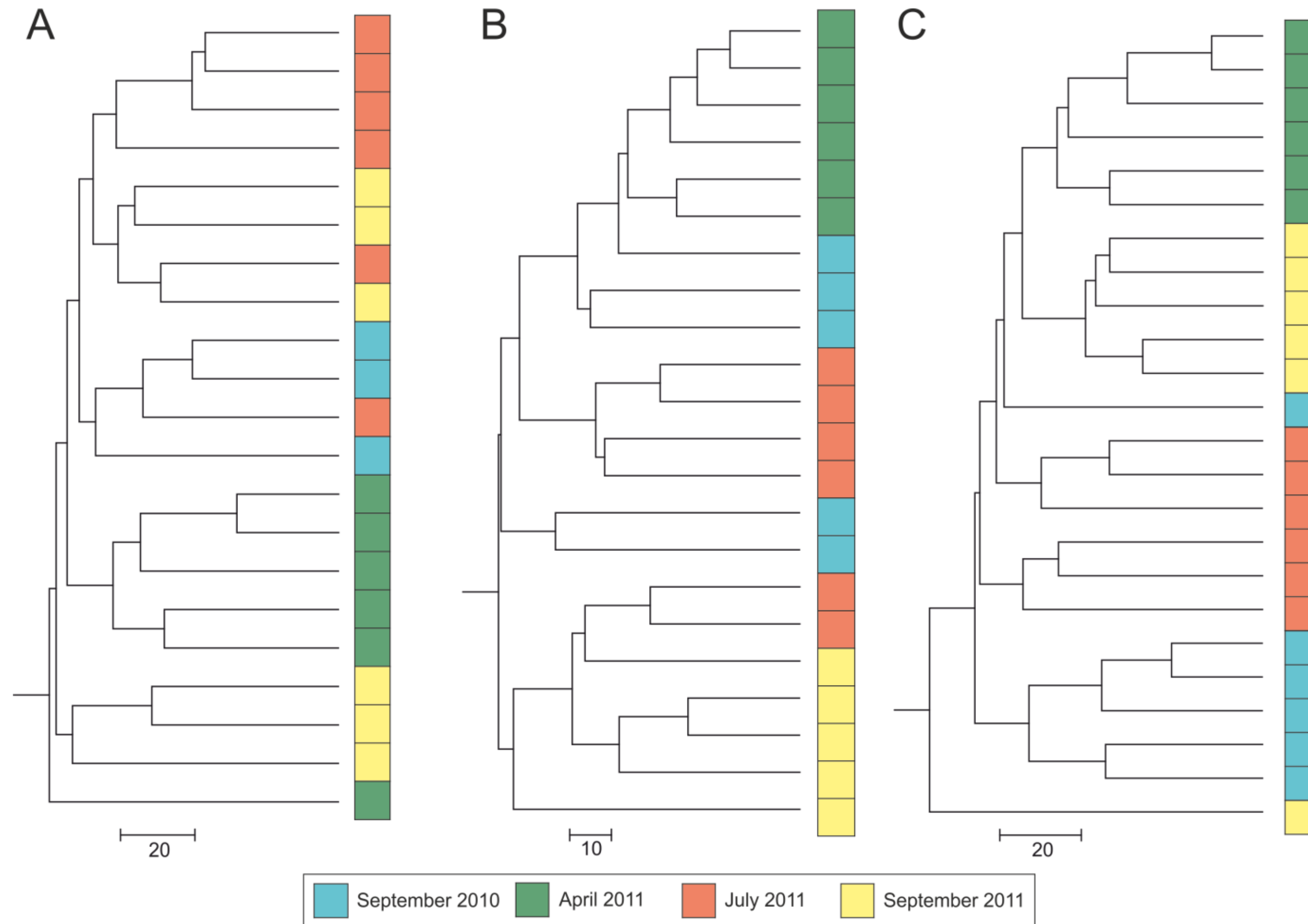

Fig. 6. UPGMA dendrogram generated by cluster analysis of DGGE fingerprints on the seasonal effect on the bacterial endophytic community composition in above-ground plant parts of *D. glomerata* (A), *F. rubra* (B), and *L. perenne* (C). For details see Fig. 3.

Moreover, three of the six samples taken in September 2010 and all samples from April 2011 formed a coherent cluster suggesting that they harbor a similar endophytic community. This distinct cluster pattern might be explained by seed transfer although this mechanism was not specifically tested in our study. It is known for some perennial plant species that several bacterial endophytes are seed-borne. These species are transferred from one plant generation to the next through the seeds of many plant species such as tobacco (Mastretta *et al.*, 2009), rice (Hardoim *et al.*, 2012), or Norway spruce (Cankar *et al.*, 2005). In a study of endophytic bacteria in switchgrass, some bacterial species were found in plants that originated from seeds sampled a year earlier (Gagne-Bourgue *et al.*, 2013). The authors regarded this as evidence for a vertical transmission to the next generation within this host plant.

Cluster analysis of the bacterial endophytic community in *L. perenne* revealed a clear separation of groups based on sampling year and season (Fig. 6C). Samples taken in 2011 formed a coherent cluster and exhibit a higher similarity to each other compared to samples taken in 2010. This finding suggests that the community structures in this grass species were different between both investigated years. Interestingly, samples collected in April 2011 and September 2011 were more similar to each other compared to samples taken in July 2011. This indicates that endophytic communities in *L. perenne* followed a seasonal pattern and that endophytic communities respond to changing climatic conditions.

DGGE band patters derived from *D. glomerata* samples revealed that samples taken in April 2011 clustered together, suggesting that they harbor a homogenous community composition (Fig. 6A). In accordance with *L. perenne*, three of the six samples taken in September 2011 were more similar to samples taken in April 2011. The other three samples of September 2011 were related to samples taken in July 2011. Such a pattern was already reported for *F. rubra*. Furthermore, samples taken in September 2010 were more similar to some of the samples taken in September 2011. These data suggested that the bacterial endophytic community in *D. glomerata* was less variable over consecutive years as, for example, the community in *L. perenne.* This might be explained by the higher endophytic diversity (number of OTUs) observed in *D. glomerata*

compared to *L. perenne*; the smaller the community size the stronger the impact of seasonal fluctuations of single species on community structure.

The different seasonal patterns recorded for the three grass species confirm our first hypothesis that the overall endophytic community structure is different between the three examined grass species. Moreover, statistical analysis supported our third hypothesis that the season has an effect on the bacterial endophytic community in the three grasses as both season and year significantly influenced the composition of these communities (Table 1). This result is consistent with other studies. According to McInroy and Kloepper (1995B), the bacterial endophytic population in sweet corn and cotton fluctuated seasonally. The season also influenced the bacterial endophytic community in elm (Mocali *et al.*, 2003) and in soybean (Kuklinsky-Sobral *et al.*, 2004). However, only cultivable endophytes were investigated in these studies.

During the year, plants undergo physiological changes that probably increase nutrient availability and thus bacterial diversity in the roots (Hallmann & Berg, 2006). This might also play a role for endophytic bacteria in the above-ground plant tissues and could explain the high similarity of *F. rubra* and *D. glomerata* samples from September and July 2011 compared to samples from April 2011. Tan *et al.* (2003) showed that environmental conditions strongly influenced the diazotrophic endophytic community structure in rice roots. Several factors, such as temperature or precipitation, have a direct effect on the plant physiology and thus an indirect impact on the colonization and the survival of bacteria in the endosphere (Hallmann *et al.*, 1997; Hardoim *et al.*, 2012). This might explain the fact that the endophytic community structure in *L. perenne* in spring and autumn showed a higher similarity compared to the community in summer due to higher precipitation in summer.

In conclusion, our results demonstrate that different management regimes affect certain bacterial endophyte communities in grass species. However, this influence varies between the applied management regimes as the effect of the fertilizer application is clearer visible compared to the impact of different mowing frequencies. In addition, the influence of the management regimes can alter with time as seasonal changes also have an impact on the endophytic community composition.

Interestingly, the effect of different management regimes and season is dependent on the host species as differences between the three investigated grass species were recorded. So far, the majority of the studies examined the effect of only one management regime in one single year, or focused on culturable endophytes or one functional group only. This study provides first insights into structural changes of endophyte communities in three agricultural important grass species as response to combined fertilizer application and mowing regimes as well as season. More studies targeting the influence of management regimes in combination with the impact of season and plant species are required to unravel the diversity of interactions between endophytic bacteria, plant species and management regimes.

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References

Araujo WL, Marcon J, Maccheroni W, Jr., Van Elsas JD, Van Vuurde JW & Azevedo JL (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and Environmental Microbiology* 68: 4906-4914.

Bacon C & Hinton D (2006). Bacterial endophytes: The endophytic niche, its occupants, and its utility. *Plant-Associated Bacteria* (Gnanamanickam S, ed), pp. 155-194. Springer, Netherlands.

Bacon C & Hinton D (2011). *Bacillus mojavensis*: Its Endophytic Nature, the Surfactins, and Their Role in the Plant Response to Infection by *Fusarium verticillioides*. *Bacteria in Agrobiology: Plant Growth Responses* (Maheshwari DK, ed), pp. 21-39. Springer Berlin Heidelberg, Germany.

Baldani J, Baldani V, Seldin L & Döbereiner J (1986). Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *International Journal of Systematic Bacteriology* 36: 86-93.

Cankar K, Kraigher H, Ravnikar M & Rupnik M (2005). Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). *FEMS Microbiology Letters* 244: 341-345.

Caporaso JG, Kuczynski J, Stombaugh J, *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336.

Chelius MK & Triplett EW (2001). The Diversity of Archaea and Bacteria in Association with the Roots of *Zea mays* L. *Microbial Ecology* 41: 252-263.

Compant S, Clément C & Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* 2: 669-678.

Compant S, Duffy B, Nowak J, Clement C & Barka EA (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71: 4951-4959.

Conn VM & Franco CM (2004). Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA clones. *Applied and Environmental Microbiology* 70: 1787-1794.

Cremonesi L, Firpo S, Ferrari M, Righetti PG & Gelfi C (1997). Double-gradient DGGE for optimized detection of DNA point mutations. *Biotechniques* 22: 326-330.

Dierschke H & Briemle G (2002). *Kulturgrasland*. Eugen Ulmer, Stuttgart, Germany.

Doty S, Oakley B, Xin G, Kang J, Singleton G, Khan Z, Vajzovic A & Staley J (2009). Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis* 47: 23-33.

Fuentes-Ramírez LE, Caballero-Mellado J, Sepúlveda J & Martínez-Romero E (1999). Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. *FEMS Microbiology Ecology* 29: 117-128.

Gagne-Bourgue F, Aliferis KA, Seguin P, Rani M, Samson R & Jabaji S (2013). Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (*Panicum virgatum* L.) cultivars. *Journal of Applied Microbiology* 114: 836-853.

Garbeva P, Overbeek LS, Vuurde JW & Elsas JD (2001). Analysis of Endophytic Bacterial Communities of Potato by Plating and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA Based PCR Fragments. *Microbial Ecology* 41: 369-383.

Gottel NR, Castro HF, Kerley M, *et al.* (2011) Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* **77**: 5934-5944.

Hallmann J (2006). Plant Interactions with Endophytic Bacteria. *Biotic Interactions in Plant-pathogen Associations* (Jeger MJ & Spence NJ, eds), pp. 87-119. CABI, Wallingford.

Hallmann J & Berg G (2006). Spectrum and Population Dynamics of Bacterial Root Endophytes. *Microbial Root Endophytes, Vol. 9* (Schulz BE, Boyle CC & Sieber T, eds.), pp. 15-31. Springer Berlin Heidelberg, Germany.

Hallmann J, Rodríguez-Kábana R & Kloepper JW (1999). Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biology and Biochemistry* 31: 551-560.

Hallmann J, Quadt-Hallmann A, Mahaffee WF & Kloepper JW (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* 43: 895-914.

Hallmann J, Quadt-Hallmann A, Rodríguez-Kábana R & Kloepper JW (1998). Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. *Soil Biology and Biochemistry* 30: 925-937.

Hardoim PR, Hardoim CCP, van Overbeek LS & van Elsas JD (2012). Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages. *PLoS ONE* **7**: e30438.

Keuter A, Hoeft I, Veldkamp E & Corre M (2013). Nitrogen response efficiency of a managed and phytodiverse temperate grassland. *Plant Soil* 364: 193-206.

Kobayashi DY & Palumbo JD (2000). Bacterial endophytes and their effects on plants and uses in agriculture. *Microbial endophytes* (Bacon CW,White JF, eds), pp. 199-236. *Marcel Dekker, New York*.

Krechel A, Faupel A, Hallmann J, Ulrich A & Berg G (2002). Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood. *Canadian Journal of Microbiology* 48: 772-786.

Kuklinsky-Sobral J, Araújo W, Mendes R, Pizzirani-Kleiner A & Azevedo J (2005). Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant and Soil* **273**: 91-99.

Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA & Azevedo JL (2004). Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology* 6: 1244-1251.

Maksimov IV, Abizgil'dina RR & Pusenkova LI (2011). Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens. *Applied Biochemistry and Microbiology* 47: 333-345.

Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J, Weyens N & Vangronsveld J (2009). Endophytic bacteria from seeds of *Nicotiana tabacum* can reduce cadmium phytotoxicity. *International Journal of Phytoremediation* 11: 251-267.

McInroy J & Kloepper J (1995A). Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil* 173: 337-342.

McInroy JA & Kloepper JW (1995B). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Canadian Journal of Microbiology* 41: 895-901.

Mocali S, Bertelli E, Di Cello F, Mengoni A, Sfalanga A, Viliani F, Caciotti A, Tegli S, Surico G & Fani R (2003). Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Research in Microbiology* 154: 105-114.

Muyzer G, de Waal EC & Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G, Teske A, Wirsen CO & Jannasch HW (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archieves of Microbiology* 164: 165-172.

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W & Backhaus H (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* 178: 5636-5643.

Olivares F, Baldani VD, Reis V, Baldani J & Döbereiner J (1996). Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae. *Biology and Fertility of Soils* 21: 197-200.

Parish R, Turkington R & Klein E (1990). The influence of mowing, fertilization, and plant removal on the botanical composition of an artificial sward. *Canadian Journal of Microbiology* 68: 1080-1085.

Petersen U, Wrage N, Köhler L, Leuschner C & Isselstein J (2012). Manipulating the species composition of permanent grasslands – A new approach to biodiversity experiments. *Basic and Applied Ecology* 13: 1-9.

Prakamhang J, Minamisawa K, Teamtaisong K, Boonkerd N & Teaumroong N (2009). The communities of endophytic diazotrophic bacteria in cultivated rice (*Oryza sativa* L.). *Applied Soil Ecology* 42: 141-149.

Quadt-Hallmann A, Kloepper JW & Benhamou N (1997). Bacterial endophytes in cotton: mechanisms of entering the plant. *Canadian Journal of Microbiology* 43: 577-582.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J & Glöckner FO (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41: D590-D596.

Reinhold-Hurek B & Hurek T (1998). Life in grasses: diazotrophic endophytes. *Trends in Microbiology* 6: 139-144.

Rosenblueth M & Martinez-Romero E (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions* 19: 827-837.

Schulz B, Wanke U, Draeger S & Aust HJ (1993). Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research* 97: 1447-1450.

Seghers D, Wittebolle L, Top EM, Verstraete W & Siciliano SD (2004). Impact of agricultural practices on the *Zea mays* L. endophytic community. *Applied and Environmental Microbiology* 70: 1475-1482.

Senthilkumar M, Anandham R, Madhaiyan M, Venkateswaran V & Sa T (2011). Endophytic Bacteria: Perspectives and Applications in Agricultural Crop Production. *Bacteria in Agrobiology: Crop Ecosystems* (Maheshwari DK, ed), pp. 61-96. Springer Berlin Heidelberg, Germany.

Sessitsch A, Reiter B, Pfeifer U & Wilhelm E (2002). Cultivation-independent population analysis of bacterial endophytes in three potato varieties based

on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. *FEMS Microbiol Ecology* 39: 23-32.

Siddiqui IA & Shaukat SS (2003). Endophytic bacteria. Prospects and opportunities for the biological control of plant-parasitic nematodes. *Nematologia Mediterranea* 31: 110-120.

Stoltzfus JR, So R, Malarvithi PP, Ladha JK & de Bruijn FJ (1997). Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant and Soil* 194: 25-36.

Sturz AV & Nowak J (2000). Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Applied Soil Ecology* 15: 183-190.

Sturz AV, Christie BR & Nowak J (2000). Bacterial endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant Sciences* 19: 1-30.

Sun L, Qiu F, Zhang X, Dai X, Dong X & Song W (2008) Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microbial Ecology* 55: 415-424.

Tan Z, Hurek T & Reinhold-Hurek B (2003) Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. *Environmental Microbiology* 5: 1009-1015.

Wilmotte A, Van der Auwera G & De Wachter R (1993) Structure of the 16 S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF ('*Mastigocladus laminosus* HTF') strain PCC7518, and phylogenetic analysis. *FEBS Letters* 317: 96-100.

SUPPORTING INFORMATION

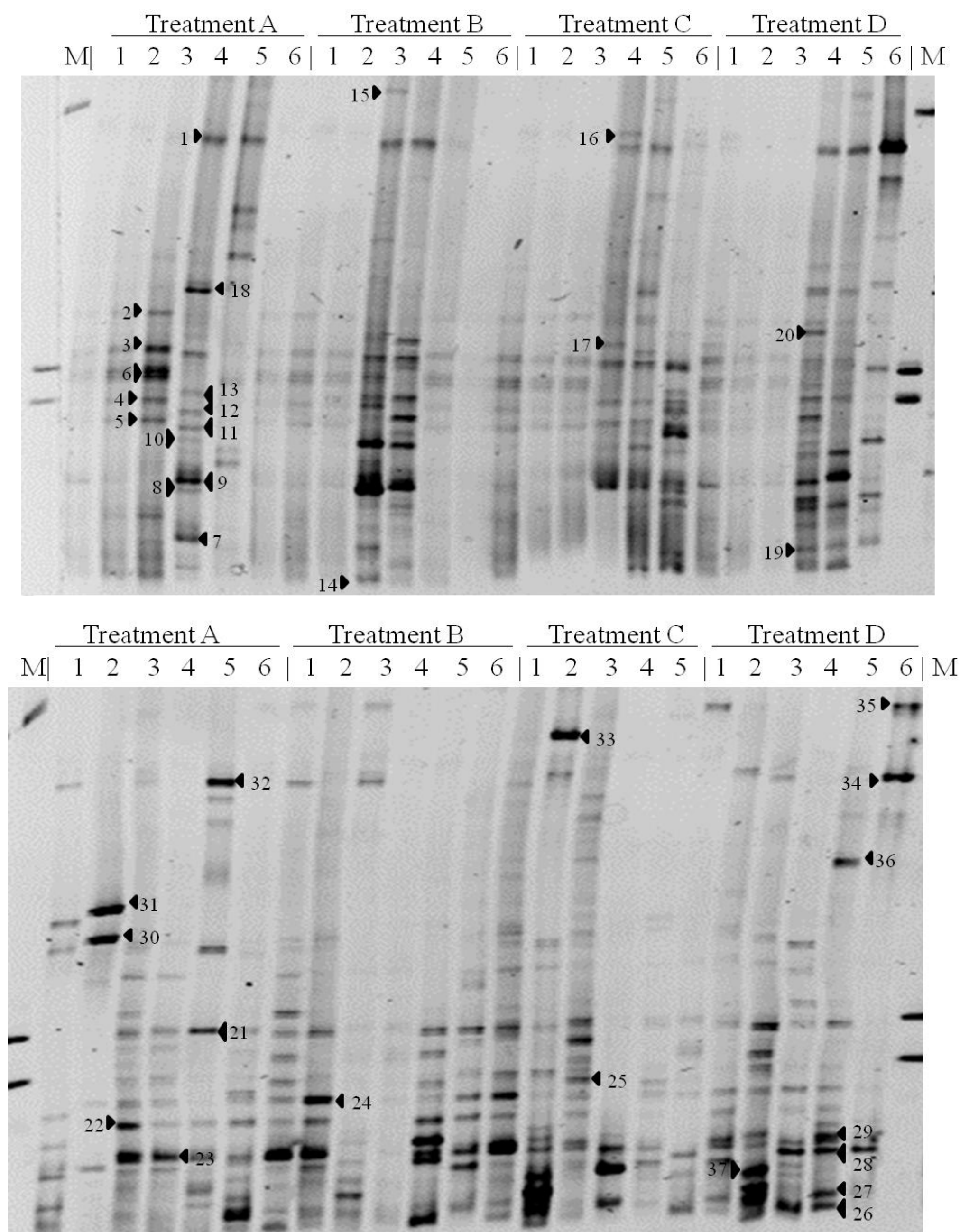

Fig. S1. 16S-DGGE profile showing the influence of different fertilization and mowing regimes on bacterial endophyte communities in above-ground plant parts of *D. glomerata*. Plant samples were taken in September 2010 (above) and 2011 (below). Independent replicates are indicated with numbers from 1 to 6. Treatment A: 1 x mowing/ year, no NPK; treatment B: 3 x mowing/ year, no NPK; treatment C: 1 x mowing/ year, NPK; treatment D: 3 x mowing/ year, NPK. M: GeneRuler *1 kb* DNA *Ladder* (Fermentas, St. Leon-Rot, Germany). Excised bands are labelled with numbers and letters, respectively.

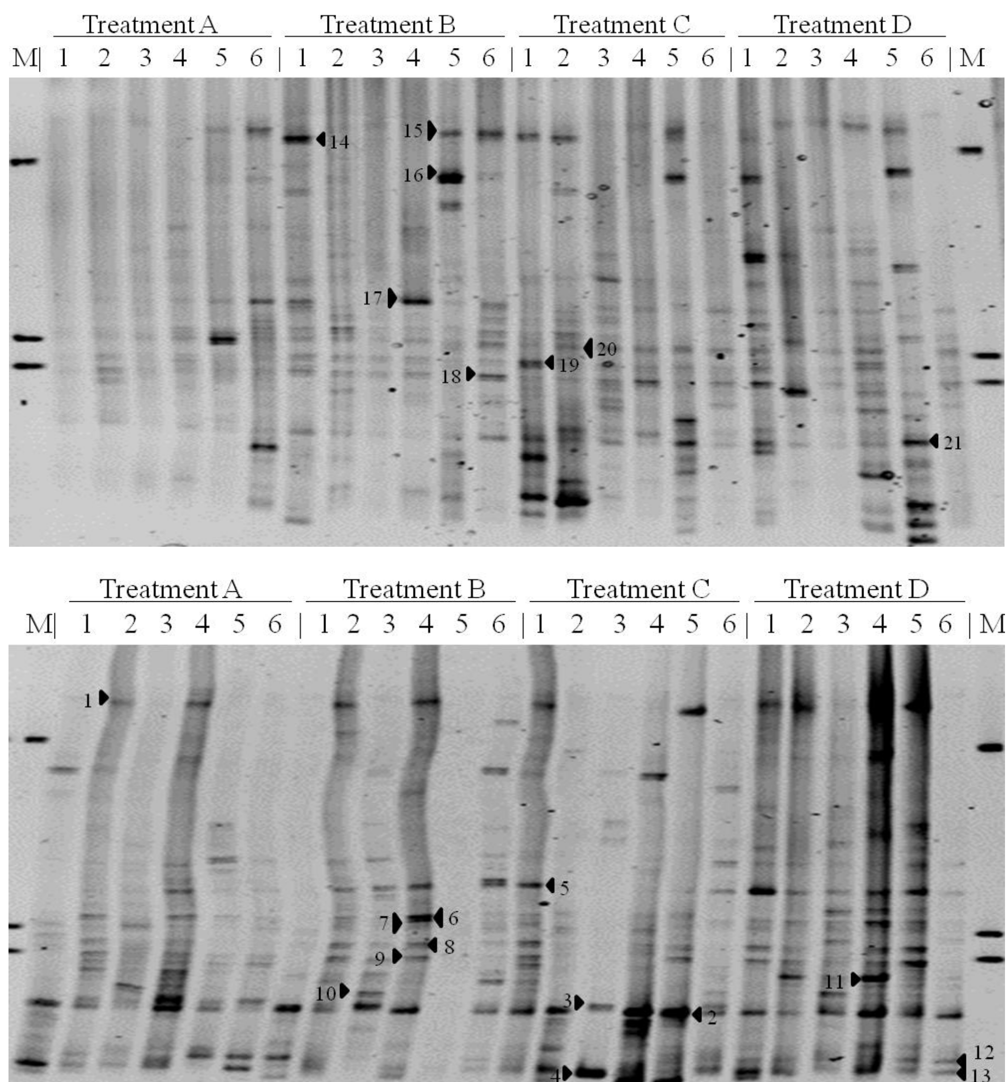

Fig. S2. 16S-DGGE profile showing the influence of different fertilization and mowing regimes on bacterial endophyte communities in aerial plant parts of *F. rubra*. Plant samples were taken in September 2010 (above) and 2011 (below). Excised bands are labelled with numbers and letters, respectively. For details see Fig. S1.

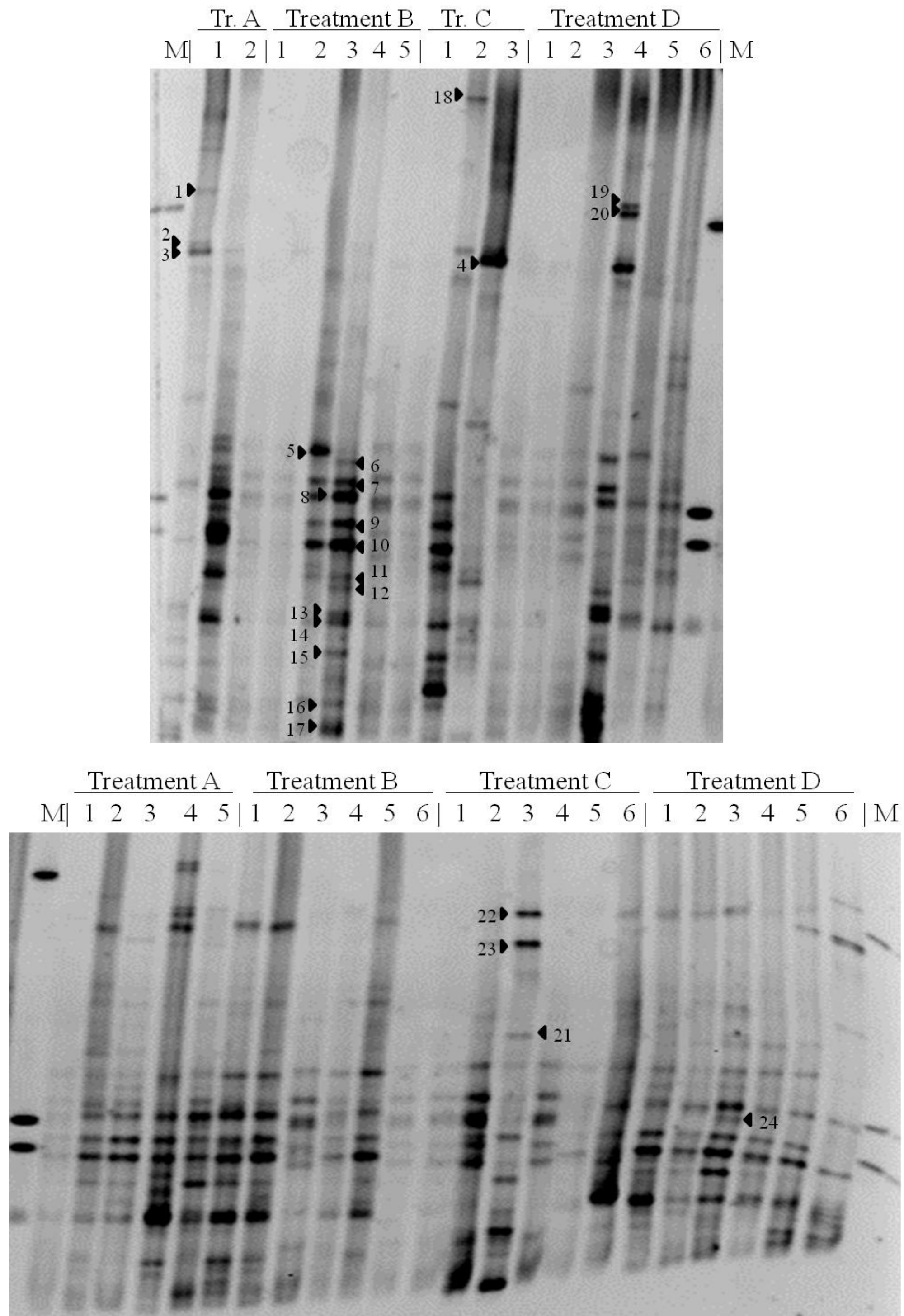

Fig. S3. 16S-DGGE profile showing the influence of different fertilization and mowing regimes on bacterial endophyte communities in above-ground plant parts of *L. perenne*. Plant samples were taken in September 2010 (above) and 2011 (below). Excised bands are labelled with numbers and letters, respectively. Tr. = Treatment. For details see Fig. S1.

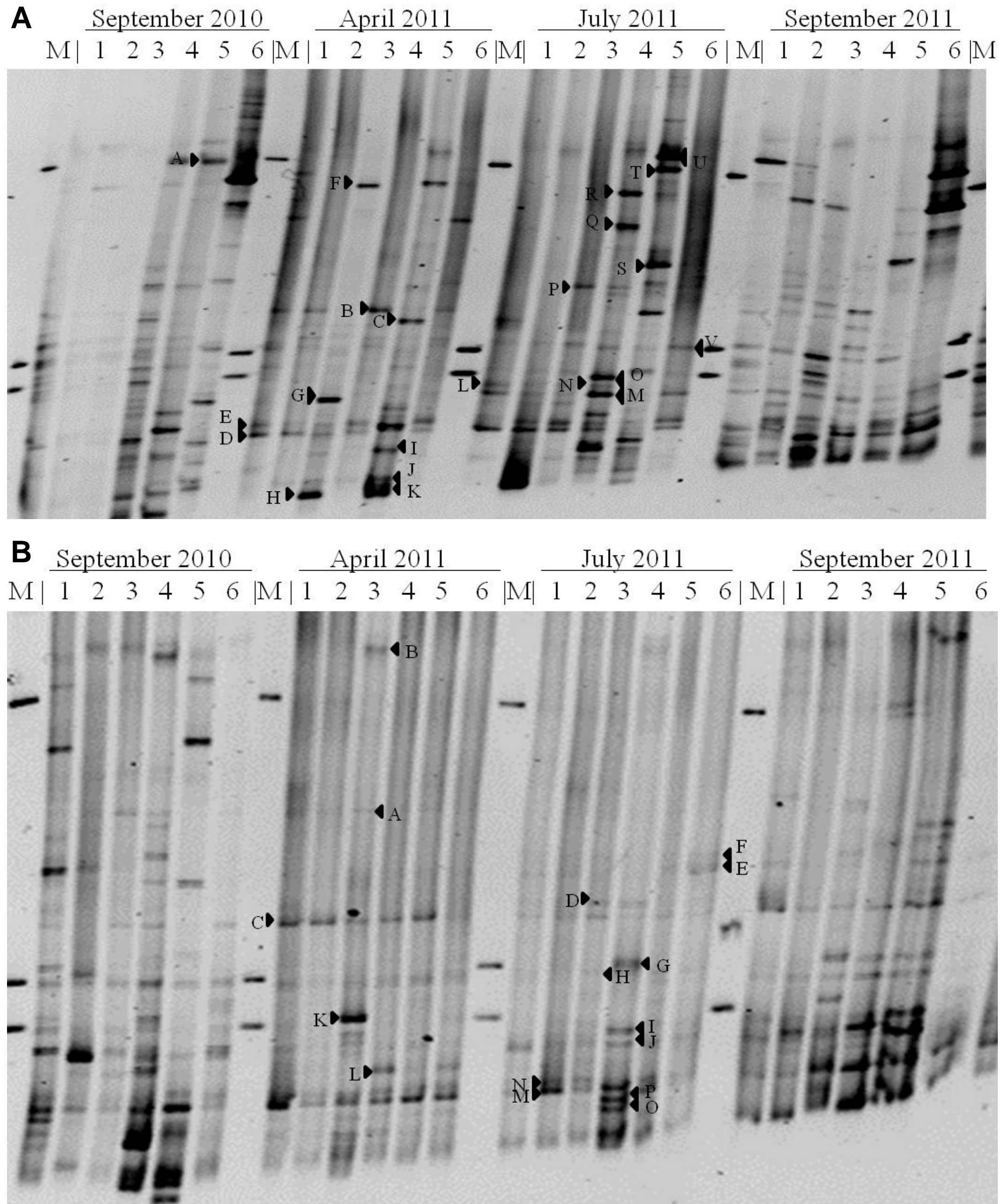

Fig. S4. 16S-DGGE profile showing the seasonal effect on bacterial endophyte communities in above-ground plant parts of *D. glomerata* (A), *F. rubra* (B), and *L. perenne* (C). Independent replicates are indicated with numbers from 1 to 6. M: GeneRuler *1 kb* DNA *Ladder* (Fermentas, St. Leon-Rot, Germany). Excised bands are labelled with numbers and letters, respectively. Samples were taken on fertilized plots three times a year in September 2010 as well as in April, July, and September 2011.

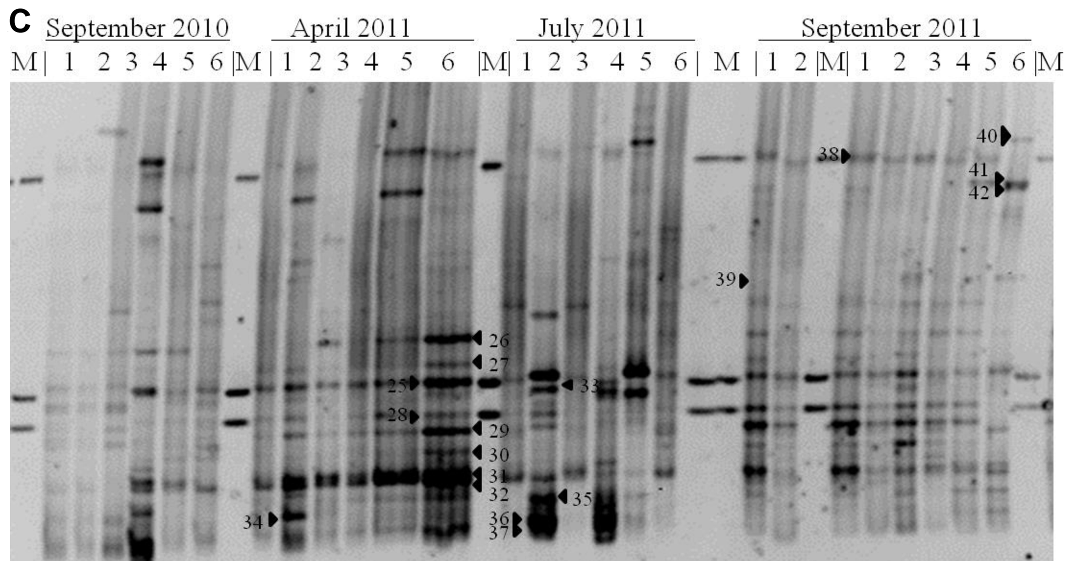

Fig. S4. continued

Table S1: Overview about all bacterial OTUs obtained by the analysis of 16S rRNA data sets derived from the isolation and the DGGE analysis.

ID		*Sequences affiliated*			*Closest hit in the SILVA database*		
	Dactylis	*Festuca*	*Lolium*	*Isolates*	*acession*	*e value*	*SILVA taxonomy*
1	1	0	0	0	HQ598842	0	*Acidobacteria; Acidobacteria; Subgroup 3; Family IncertaeSedis; Bryobacter; uncultured Acidobacteria bacterium*
2	0	0	1	0	JF176919	0	*Actinobacteria; Acidimicrobiia; Acidimicrobiales; Acidimicrobiaceae; CL500-29 marine group; uncultured bacterium*
3	0	0	0	1	KC236620	0	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Curtobacterium; Curtobacterium sp. 4136*
4	0	0	1	0	Y17233	0	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium; Microbacteriumkeratanolyticum*
5	1	0	1	0	KC169799	0	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium; Microbacterium sp. CC-AMFLN-3*
6	1	0	0	0	Y17240	1E-168	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Microbacterium; Microbacteriumtrichothecenolyticum*
7	0	0	0	1	JX133202	0	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Plantibacter; Plantibactercousiniae*
8	0	0	0	1	JQ071511	0	*Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Micrococcus; Micrococcus yunnanensis*
9	1	0	0	0	AB672179	0	*Actinobacteria; Thermoleophilia; Gaiellales; uncultured; uncultured bacterium*
10	1	0	0	0	JX091739	0	*Chloroflexi; S085; uncultured bacterium*
11	1	0	0	0	AM696939	2E-158	*Deinococcus-Thermus; Deinococci; Deinococcales; Trueperaceae; Truepera; uncultured bacterium*
12	1	0	0	0	AB374378	0	*Deinococcus-Thermus; Deinococci; Deinococcales; Trueperaceae; Truepera; uncultured endolithic bacterium*
13	1	0	0	0	KC120646	0	*Deinococcus-Thermus; Deinococci; Thermales; Thermaceae; Thermus; uncultured bacterium*
14	0	0	0	3	KC441733	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus licheniformis*
15	0	0	0	1	KC434960	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus safensis*
16	0	0	0	1	KC434960	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus safensis*
17	0	0	0	2	KC310814	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus sp. A8(2013)*
18	0	0	0	1	FN395277	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus sp. FR-W2C1*
19	0	0	0	3	KC441785	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus subtilis*
20	0	0	0	11	JX436372	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Firmicutes bacterium Man17*
21	0	0	2	0	HE974809	0	*Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; uncultured Bacillus sp.*
22	0	0	0	2	EU282459	0	*Firmicutes; Bacilli; Bacillales; Family XII IncertaeSedis; Exiguobacterium; Exiguobacterium sp. TC38-2b*
23	0	0	0	1	AB363733	0	*Firmicutes; Bacilli; Bacillales; Paenibacillaceae; Paenibacillus; Paenibacilluslautus*
24	0	0	0	10	JX897938	0	*Firmicutes; Bacilli; Bacillales; Paenibacillaceae; Paenibacillus; Paenibacillusxylanexedens*
25	0	0	0	1	FM173819	0	*Firmicutes; Bacilli; Bacillales; Paenibacillaceae; Paenibacillus; Pseudomonas sp. CL4.14*
26	0	0	0	1	JX990163	0	*Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus; Bacillales bacterium Cul_0304*
27	0	0	0	1	JX898015	0	*Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus; Bacillus sp. FBst09*
28	0	0	0	1	JN208189	0	*Firmicutes; Bacilli; Bacillales; Planococcaceae; Lysinibacillus; Lysinibacillus sp. DT3*
29	0	0	0	1	JX996174	0	*Firmicutes; Bacilli; Bacillales; Planococcaceae; Solibacillus; Solibacillussilvestris*
30	0	0	0	1	JX996174	0	*Firmicutes; Bacilli; Bacillales; Planococcaceae; Solibacillus; Solibacillussilvestris*
31	3	5	1	0	X70648	0	*Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; Staphylococcus aureus*
32	1	2	1	0	L37605	0	*Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; Staphylococcus epidermidis*
33	1	1	0	0	KC153285	0	*Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; Staphylococcus sp. G2-10*
34	0	0	0	1	KC012992	0	*Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; Staphylococcus sp. JP44SK55*
35	1	0	0	0	HQ792508	0	*Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus; uncultured organism*
36	0	1	0	0	JQ901473	0	*Firmicutes; Clostridia; Clostridiales; Family XI IncertaeSedis; Peptoniphilus; uncultured bacterium*
37	0	0	0	1	KC003398	0	*Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia; unidentified marine bacterioplankton*
38	0	2	0	0	JN713899	0	*Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Tepidimonas; Tepidimonas sp. AT-A2*

Table S1 continued.

ID		Sequences affiliated			Closest hit in the SILVA database		
	Dactylis	Festuca	Lolium	Isolates	acession	e value	SILVA taxonomy
39	1	2	1	0	JX271982	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; uncultured; uncultured bacterium
40	1	0	0	0	HQ222272	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; uncultured; Variovorax sp. enrichment culture clone Van40
41	0	1	0	0	KC286834.	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Variovorax; uncultured bacterium
42	0	1	0	0	X74914	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Duganella; Zoogloearamigera
43	0	1	0	0	Y10146	2E-129	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Herbaspirillum; Herbaspirillumseropedicae
44	1	2	1	0	Y08846	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Janthinobacterium; Janthinobacteriumlividum
45	0	1	2	0	JN024091	0	Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Massilia; uncultured bacterium
46	0	0	1	0	JQ278953	0	Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; uncultured; uncultured beta proteobacterium
47	0	2	0	0	KC331513	0	Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Azospira; uncultured bacterium
48	1	0	1	0	Z96082	0	Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Pantoea; Pantoeaagglomerans
49	0	1	0	0	HQ801751	0	Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; uncultured organism
50	0	0	0	1	JX849037	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Enhydrobacter; Moraxella osloensis
51	0	1	0	0	JX849037	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Enhydrobacter; Moraxella osloensis
52	0	0	0	2	HQ178997	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; bacterium OC25(2011)
53	0	0	1	0	X99541	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas anguilliseptica
54	1	1	1	0	AF054936	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas balearica
55	1	1	0	0	KC342251	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas chlororaphis
56	1	0	0	0	Z76673	7E-114	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas chlororaphis
57	1	0	1	0	FJ976601	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas putida
58	0	0	1	0	KC310832	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas sp. C2(2013)
59	1	1	0	0	KC310832	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas sp. C2(2013)
60	0	0	1	0	JX899644	6E-174	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas sp. REm-amp_189
61	0	1	1	0	U65012	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas stutzeri
62	1	0	1	0	Z76669	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; Pseudomonas syringae
63	1	1	0	0	DQ469202	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; uncultured bacterium
64	0	0	0	5	HM261524	0	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; uncultured bacterium
65	1	0	1	0	GQ262820	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; uncultured; uncultured bacterium
66	1	0	0	0	JN023904	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; uncultured; uncultured bacterium
67	1	0	0	0	EF018613	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; uncultured; uncultured proteobacterium
68	1	1	0	0	JN872548	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Luteibacter; Xanthomonadaceae bacterium SAP40_3
69	1	0	0	0	FJ164060	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Rhodanobacter; gamma proteobacterium CH23i
70	0	1	0	0	FJ380140	2E-170	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Rhodanobacter; uncultured bacterium
71	0	0	1	0	JF180263	2E-151	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Rhodanobacter; uncultured bacterium
72	0	0	0	2	JN897284	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas; Pseudomonas poae
73	0	1	0	0	JX205209	0	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas; Pseudomonas sp. MLB-42
74	1	0	0	0	X95923	2E-155	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas; Stenotrophomonasmaltophilia

STUDY 2

Mixed effects of management regimes and herbivory on bacterial community composition in the rhizosphere of permanent grassland

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Performed the experiments: FW, DK

Analyzed data: FW, SH, BW

Wrote the publication: FW, RD, SV

Conceived and designed the experiments: FW, SV

Abstract

The plant rhizosphere is regarded as a dynamic environment in which several parameters influence the diversity, activity, and composition of bacterial communities. Despite their importance for soil and plant health, the response of these communities to different grassland management regimes and to above-ground herbivory is still poorly understood. This study aimed at assessing and exploiting the bacterial diversity in the plant rhizosphere with regard to sward composition, different fertilization and mowing regimes, as well as above-ground herbivory. For this purpose, a lysimeter experiment was conducted on a semi-natural, moderately species-rich grassland site. Following a two-week exposure to herbivory, soil samples were taken from the plant rhizosphere. Community structures were assessed by DGGE as well as large-scale pyrosequencing-based analysis of 16S rRNA gene sequences. More than 450,000 sequences were used to assess diversity and composition of bacterial communities. We recorded significant differences in bacterial diversity and richness with respect to the investigated parameters. Further analysis revealed that not only the parameters solely but also the combinations influenced the abundances of several bacterial taxa. Such combined effects led to either an enhanced, reduced, or, in rare cases, opposite bacterial response. These unique combinations of parameters studied and the high phylogenetic resolution provides exceptional insights into the diversity and ecology of bacterial communities in the plant rhizosphere. Moreover, the results of this study enable us to better validate the impact of different management regimes and herbivory on these communities and to predict potential ecological implications.

Introduction

The plant rhizosphere, defined as the soil layer surrounding the plant roots (Sørensen, 1997), is a complex and dynamic environment. Microbial communities colonizing these habitats play a major role for plant growth and health (Berg & Smalla, 2009, Compant *et al.*, 2010) as well as for functioning of fundamental processes such as nutrient cycling (Marschner *et al.*, 2004, Berg & Smalla, 2009)

or denitrification processes (Pastorelli *et al.*, 2011). Rhizospheric bacteria may form close mutualistic relationships with plants, which are important for the structure and dynamics of plant communities in almost all terrestrial ecosystems (van der Heijden *et al.*, 2008). Moreover, they may promote higher resistance to plant pathogens and parasites such as nematodes or insects (Kloepper *et al.*, 1992, Ramamoorthy *et al.*, 2001, Lugtenberg & Kamilova, 2009) and help plants to tolerate abiotic stress including salt, drought or nutrient deficiency (Dimkpa *et al.*, 2009, Yang *et al.*, 2009).

The development of culture-independent molecular approaches has significantly enhanced our understanding of bacterial communities in different environments such as rhizosphere bacteria in grassland soils (Nunan *et al.*, 2005, Singh *et al.*, 2007). One of the most frequently used techniques to explore bacterial communities in soil or rhizosphere is denaturing gradient gel electrophoresis (DGGE) (Yang & Crowley, 2000, Duineveld *et al.*, 2001, Smalla *et al.*, 2001, Nunan *et al.*, 2005, Costa *et al.*, 2006). Recently, high-throughput pyrosequencing of 16S rRNA gene fragments has been applied for in-depth analysis of these communities (Acosta-Martinez *et al.*, 2008, Gottel *et al.*, 2011, Nacke *et al.*, 2011).

The diversity, activity, and structure of bacterial communities in the rhizosphere are shaped by several parameters. Soil type or plant species are regarded as the most dominant factors (Grayston *et al.*, 1998, Duineveld *et al.*, 2001, Kowalchuk *et al.*, 2002, Garbeva *et al.*, 2008, Berg & Smalla, 2009, Gottel *et al.*, 2011). Additional important factors shaping bacterial communities in the rhizosphere are plant root exudates (Garbeva *et al.*, 2008, Haichar *et al.*, 2008), the soil pH (Marschner *et al.*, 2004), and fertilizer application (Marschner *et al.*, 2004, Doi *et al.*, 2011). A few recent studies have also examined the influence of land use and management regime on rhizosphere bacterial communities (Costa *et al.*, 2006, Garbeva *et al.*, 2008).

In addition to the parameters mentioned above, below-ground herbivory also affects bacterial communities in the rhizosphere (Denton *et al.*, 1998, Treonis *et al.*, 2005, Poll *et al.*, 2007, Dematheis *et al.*, 2012). For example, soil dwelling pests such as the western corn rootworm larvae (*Diabrotica virgifera virgifera*) or the leather jacket larvae (*Tipula paludosa*) have been shown to change the

rhizosphere bacterial community composition by feeding on the roots (Treonis *et al.*, 2005, Dematheis *et al.*, 2012). The authors suggest that these changes are linked to shifts in root exudates patterns. However, studies investigating the influence of above-ground herbivory on bacterial communities in the rhizosphere are still missing.

In this study, we investigated the bacterial community composition in the rhizosphere with regard to sward composition (monocot-reduced, dicot-reduced, and species-rich as control), different grassland management regimes (with vs. without fertilization; mown once vs. thrice per year), and above-ground herbivory. More specifically, we wanted to evaluate the impact of these four parameters on rhizospheric bacterial communities separately and in combination. Therefore, a lysimeter experiment was established on a semi-natural, moderately species-rich grassland site near Silberborn (Solling; Germany). Soil samples were collected from the lysimeters after two-weeks herbivory and further investigated employing different metagenomic approaches. To gain insights into the bacterial community composition, total DNA was extracted from the samples and subjected to 16S rRNA gene analyses. The community composition was either studied by DGGE analysis or pyrosequencing-based sequencing of 16S rRNA genes.

To our knowledge, this is the first study using two metagenomic approaches to analyze the impact of (1) sward composition, (2) fertilization, (3) different mowing frequencies, (4) above-ground herbivory on the bacterial community in the rhizosphere in one single field experiment on a permanent semi-natural grassland site.

Materials and methods

Study site

We used the Grassland Management Experiment (GrassMan) for this study, comprising different management intensity treatments. This long-term field experiment was established at a semi-natural, moderately species-rich grassland site in the Solling Mountains in Lower Saxony, central Germany (51°44'53" N, 9°32'43" E, 490 m a.s.l.) in spring 2008. The permanent grassland site has been traditionally used as an extensive pasture and meadow since the end of the 19th

century (Petersen *et al.*, 2012). The dominating soil type of the experimental area has been determined as a shallow (40-60 cm), stony Haplic Cambisol with a pH_{KCL} ranging from 4.18 to 5.47 (for details see Keuter *et al.*, 2013). The mean annual temperature at this site is 6.9°C and the mean annual precipitation 1028 mm (Deutscher Wetterdienst 1960-1990, station Silberborn-Holzminden, 440 m a.s.l.).

Experimental design

The full-factorial design of this study included two mowing frequencies (once per year in July vs. thrice per year in May, July, and September, respectively) and two fertilization treatments (no vs. NPK fertilization). The N fertilizer was applied as calcium ammonium nitrate N27 in two equal doses (180 kg N ha^{-1} yr^{-1}) in April and end of May. In addition, 30 kg P ha^{-1} yr^{-1} plus 105 kg K ha^{-1} yr^{-1} as Thomaskali® (8% P_2O_5, 15% K_2O, 20% CaO) were applied at the end of May. All plots were cut to a height of 7 cm with a Haldrup® harvester. The third factor established in this experiment was a manipulation of the sward composition (monocot-reduced, dicot-reduced, species-rich), established by selective herbicide applications to decrease either dicots (Starane® and Duplosan KV; active ingredients: Mecoprop-P® and Fluroxypyr/ Triclopyr; 3 l ha^{-1} each) or monocots (Select 240 EC® by Stähler Int., Stade, Germany; active ingredients: Clethodim; 0.5 l ha^{-1}). One third of the plots were maintained as species-rich controls. The application of herbicides took place on 31st of July 2008 resulting in significant changes in species richness and in functional group abundances (Petersen *et al.*, 2012). Each treatment was replicated six times, resulting in 72 plots of 15 x 15 m size arranged in a Latin rectangle (for further details see Petersen *et al*. 2012).

Additionally, a lysimeter experiment was established with two lysimeters per plot in August and September 2010. The lysimeters consisted of a transparent plexiglass tube (diameter 14.4 cm, length 30 cm), which contained the original and intact soil core. The tubes were installed without damaging the vegetation and the soil core; they were slowly pushed downwards into the soil by applying hydraulic pressure. Drainage water was collected in a PE bottle that was placed underneath all lysimeters. One lysimeter per plot was used as herbivory lysimeter; the other one was left as control lysimeter. Four adult female grasshoppers

(*Chorthippus* spec.) and two Roman snails (*Helix pomatia* L.) per herbivory lysimeter and plot were applied. Cages for the herbivores were built of gauze of 1.5 mm mash size and were fixed on the top of the lysimeter. The experiments were started in August 2011, and were run for two weeks.

Sample collection, pH measurement, and DNA extraction

After two-weeks herbivory, soil samples were taken in autumn 2011. For this purpose, the lysimeter core was harvested, the above-ground vegetation was removed, and the top 5 cm of the soil core were homogenized. Coarse roots and stones (>5 mm) were subsequently removed. Soil samples were immediately cooled down (below 4°C), transported to the laboratory and kept frozen at -80°C until further use.

To measure the soil pH, 2 g of soil per lysimeter were mixed with 5 ml 1 M KCl. The pH was determined after 12 h incubation time (Supplemental Tab. S1). As soil pH can influence the bacterial community structures in rhizosphere (Marschner *et al.*, 2004) and measured pH values were inhomogeneous over the research area, we initially tested for correlation between pH and the four studied parameters (sward composition, fertilization, mowing frequency, and above-ground herbivory). No significant correlation was found.

Environmental DNA was extracted employing the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The lysimeter samples of at least three plots (DGGE 4, pyrosequencing 3 samples) per treatment were used for DNA extraction and further analysis. The samples were analyzed by DGGE as well as large-scale pyrosequencing-based analysis of 16S rRNA gene sequences.

Amplification of the 16S rRNA genes for DGGE analysis

PCR amplification targeting the V6-V8 region of the 16S rRNA gene was performed with the primers F968-GC (5'-AACGCGAAGAACCTTAC-3') and R1401 (5'-CGG TGTGTACAAGACCC-3') (Nübel *et al.*, 1996, Zoetendal *et al.*, 2002). In order to prevent complete denaturation of the fragment, a GC-rich sequence (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3') was attached at the 5'-end of the primer F968-GC (Muyzer *et al.*, 1993).

The PCR reaction mixture (25 µl) for amplification of the target gene contained 2.5 µl of 10-fold Mg-free Taq polymerase buffer (Fermentas, St. Leon-Rot, Germany), 200 µM of each of the four deoxynucleoside triphosphates, 1.75 mM $MgCl_2$, 0.4 µM of each primer, 5% DMSO, 1 U of Taq DNA polymerase (Fermentas), and approximately 25 ng of the DNA sample as template. Negative controls were performed by using the reaction mixture without template. Three independent PCR reactions were performed and obtained PCR products were pooled in equal amounts. The following thermal cycling scheme was used: initial denaturation at 94°C for 5 min, 11 cycles of: 1 min at 94°C, 1 min at 60°C (minus 1°C per cycle) and 2 min at 72°C, followed by 17 cycles of: 1 min at 94°C, 1 min at 53°C and 2 min at 72°C. The final extension was carried out at 72°C for 10 min. The resulting PCR products were checked for appropriate size by agarose gel electrophoresis.

Denaturing Gradient Gel Electrophoresis (DGGE)

The DGGE analyses of the bacterial communities were performed by using a PhorU2 apparatus (Ingeny, Goes, the Netherlands) with a double gradient. The first gradient ranged from 55 to 68% denaturant with an additional gradient of 6.2 to 9% acrylamide. This enhances the bands' sharpness and resolution (Cremonesi *et al.*, 1997). The denaturant (100%) contained 7 M urea and 40% formamide. Approximately 100 ng of the pooled PCR product were loaded on the gel. For each treatment, at least three independent DGGE were performed. The run was performed in Tris-acetate-EDTA buffer (40 mM Tris, 20 mM NaAcetate, 1 mM Na_2EDTA, pH 7.4) at 60°C. After electrophoresis for 16 h at 100 V, the gels were stained for 45 min with SYBRGold (Invitrogen, Carlsbad, USA). The stained DGGE gels were immediately photographed on a UV trans-illumination table.

DGGE data analysis and statistical testing

Analysis of DGGE profiles was performed using the software package GELCOMPAR II, version 5.1 (Applied Math, Ghent, Belgium). Cluster analyses (UPGMA) based on Pearson correlation were performed to evaluate the percentage of similarity shared among the samples from the different treatments.

Community analysis using pyrosequencing

To analyze the bacterial diversity, the V3-V5 region of the bacterial 16S rRNA was amplified by PCR. The PCR reaction (25 µl) contained 5 µl of 5-fold Phusion GC buffer (Finnzymes, Vantaa, Finland), 200 µM of each of the four desoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 4 µM of each primer (see below), 2.5% DMSO, 1 U of Phusion High Fidelity Hot Start DNA polymerase (Finnzymes), and approximately 25 ng of extracted DNA. The following thermal cycling scheme was used: initial denaturation at 98°C for 5 min, 25 cycles of denaturation at 98°C for 45 s, annealing at 68°C for 45 s, followed by extension at 72°C for 30 s. The final extension was carried out at 72°C for 5 min. Negative controls were performed by using the reaction mixture without template.

The V3-V5 region was amplified with the following set of primers according to Muyzer *et al.* (1995) containing the Roche 454 pyrosequencing adaptors, keys, and one unique MID per sample (underlined): V3for (341f) 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-$(dN)_{10}$-CCTACGGGAGGCAGCAG-3′ and V5rev (907r) 5′- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-CCGTCAATTCCTTTRAGTTT-3′. The resulting PCR products were checked for appropriate size and purified employing the peqGOLD Gel Extraction Kit (Peqlab) as recommended by the manufacturer.

Quantification of the PCR products was performed using the Quant-iTdsDNAHS assay kit and a Qubit fluorometer (Invitrogen) as recommended by the manufacturer. Three independent PCR reactions were performed per sample and the obtained PCR products were pooled in equal amounts. The Göttingen Genomics Laboratory determined the sequences of the 16S rRNA by using a Roche GS-FLX+ 454 pyrosequencer with Titanium chemistry (Roche, Mannheim, Germany).

Generated 16S rRNA datasets were processed and analyzed according to Wemheuer *et al.* (2014). In summary: after raw data extraction, pyrosequencing reads shorter than 250 bp, with an average quality value below 25, or possessing long homopolymer stretches (> 8 bp) were removed. Afterwards, the sequences were denoised. Chimeric sequences were subsequently removed using UCHIME (Edgar *et al.*, 2011) and the most recent Greengenes CoreSet (DeSantis *et al.*, 2006) as reference dataset. Processed sequences of all samples were joined, sorted

by decreasing length, and clustered employing the UCLUST algorithm (Edgar, 2010) implemented in the QIIME software package.

Sequences were clustered in operational taxonomic units (OTUs) at 1%, 3%, and 20% genetic dissimilarity. Phylogenetic composition was determined using the QIIME assign_taxonmy.py script. A BLAST alignment against the Silva SSURef 111 NR database (Pruesse *et al.*, 2007) was thereby performed. Sequences were classified with respect to the silva taxonomy of their best hit. Rarefaction curves, Shannon indices, ACE indices, and Chao1 indices were calculated employing QIIME. In addition, the maximal number of OTUs (n_{max}) was estimated for each sample using the Michaelis-Menten-fit alpha diversity metrics included in the QIIME software package. To compare bacterial community structures across all samples based on phylogenetic or count-based distance metrics, Principal Coordinate Analyses (PCoA) were generated using QIIME. A phylogenetic tree was calculated prior to PCoA generation. For this purpose, sequences were aligned using the PyNAST algorithm implemented in the QIIME software package. The phylogenetic tree and the respective OTU table were subsequently used to calculate PCoAs.

Statistical analyses

Statistical analyses were performed employing R (RDevelopmentCoreTeam, 2012; Version 2.15.0). To validate the impact of the different management regimes and herbivory on the measured soil pH as well as on the diversity indices, an Analysis of Variance (ANOVA) was performed. The effects of the different treatments on relative abundances of predominant bacterial groups were tested by Dirichelet regression in R using the DirichletReg package. Either the most abundant bacterial phyla and proteobacterial classes or the abundant (> 0.1%) OTUs at 3% genetic divergence were used for in this analysis.

Results

General analyses of the pyrosequencing-derived dataset

To fully assess the bacterial community structures, we applied amplicon-based pyrosequencing. A total of 468,538 high-quality bacterial 16S rRNA gene

sequences with an average read length of 504 bp were used for the community analyses. The number of sequences per sample ranged from 2,291 to 12,795. All sequences could be classified below phylum level. Rarefaction curves, richness, and alpha diversity indices were calculated at 1, 3, 20% genetic distance using 2,280 randomly selected sequences per sample. At 20% sequence divergence, most rarefaction curves reached saturation, indicating that the surveying effort covered almost the full extent of taxonomic diversity at this genetic distance (Supplemental Fig. S3C). The calculated coverage varied between 71.81 and 87.63% (Supplemental Tab. S2). At 3 and 1% genetic distance, the rarefaction curves were not saturated (Supplemental Fig. 3A and B). The calculated coverage was between 30.40 and 72.59% (3% genetic distance) and between 25.50 and 71.74% (1% genetic distance) (Supplemental Tab. S2). For all samples, the Shannon index of diversity (H') was determined (Supplemental Tab. S2). The Shannon index ranged from 2.65 to 3.51, from 4.94 to 6.1, and from 5.29 to 6.34 at a genetic distance of 20, 3, and 1%, respectively.

Characterization of bacterial community structure in the rhizosphere

Sequences were mainly affiliated to 7 phyla and 4 proteobacterial classes (Fig. 1 and 2, and Supplemental Tab. S3). The dominant phyla and proteobacterial classes across all samples were *Acidobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Chloroflexi*, representing 24.63, 21.77, 16.16, 7.27, 6.18, 5.59, 4.72, 3.59, 2.98, 2.97%, respectively. These phylogenetic groups were present in all samples. The three dominant phyla *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* represented approximately 84% of all classified sequences. Other bacterial phyla were less abundant (<1% of all classified sequences) (Fig. 2, Supplemental Tab. S4). The members of these rare phyla included, i.e., *Chlorobi*, *Nitrospirae*, *Fibrobacteres*, *Verrucomicrobia*, *Cyanobacteria*, *Spirochaetes*, *Planctomyces*, *Fusobacteria*, and *Deinococcus-Thermus*.

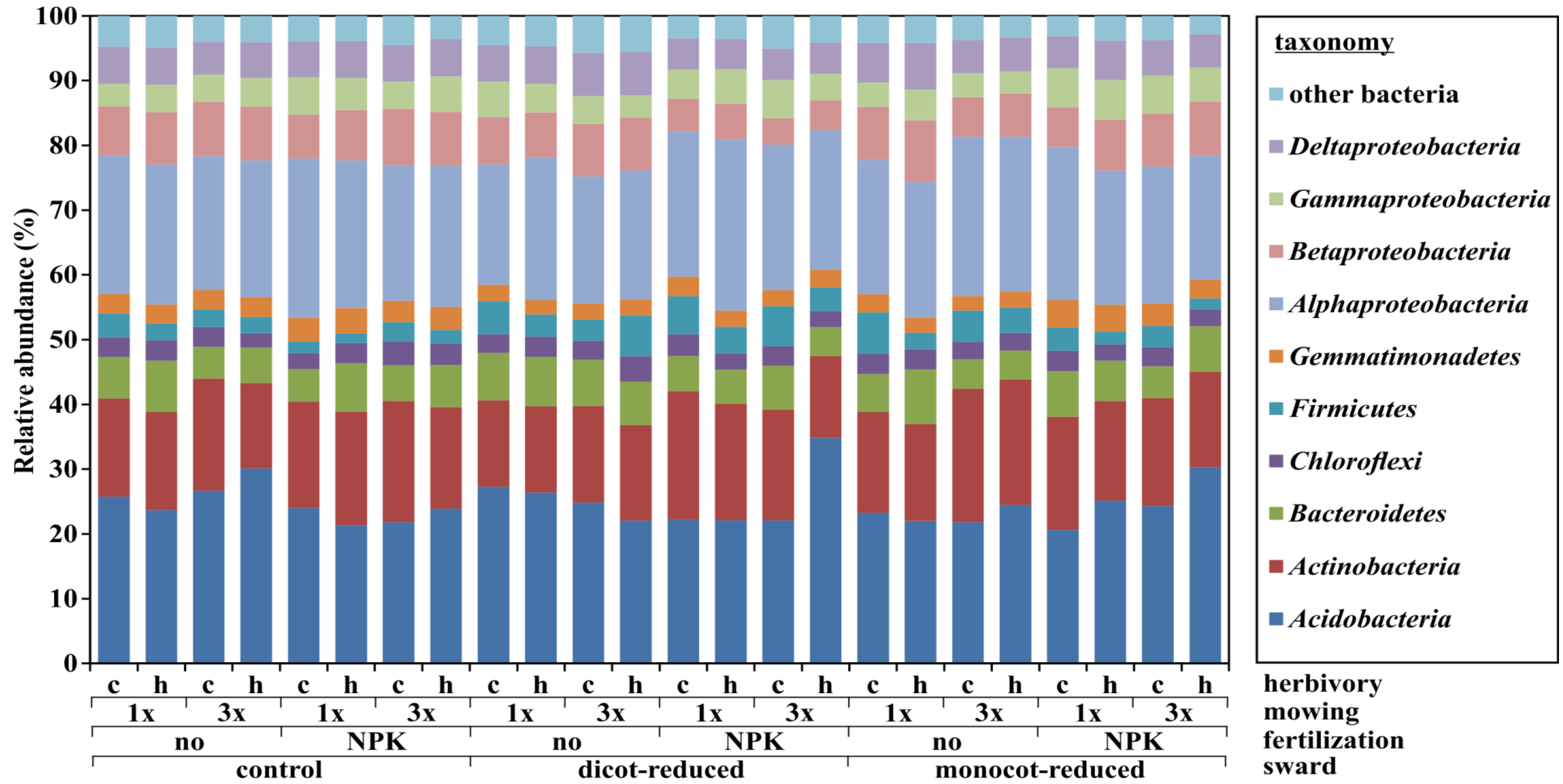

Fig. 1. Relative abundances of different predominant bacterial phyla and proteobacterial classes as revealed by pyrosequencing-based analysis of generated 16S rRNA gene amplicons. Abundances are mean values of the three replications per treatment. Only phyla and proteobacterial classes with more than 1% mean abundance are shown.

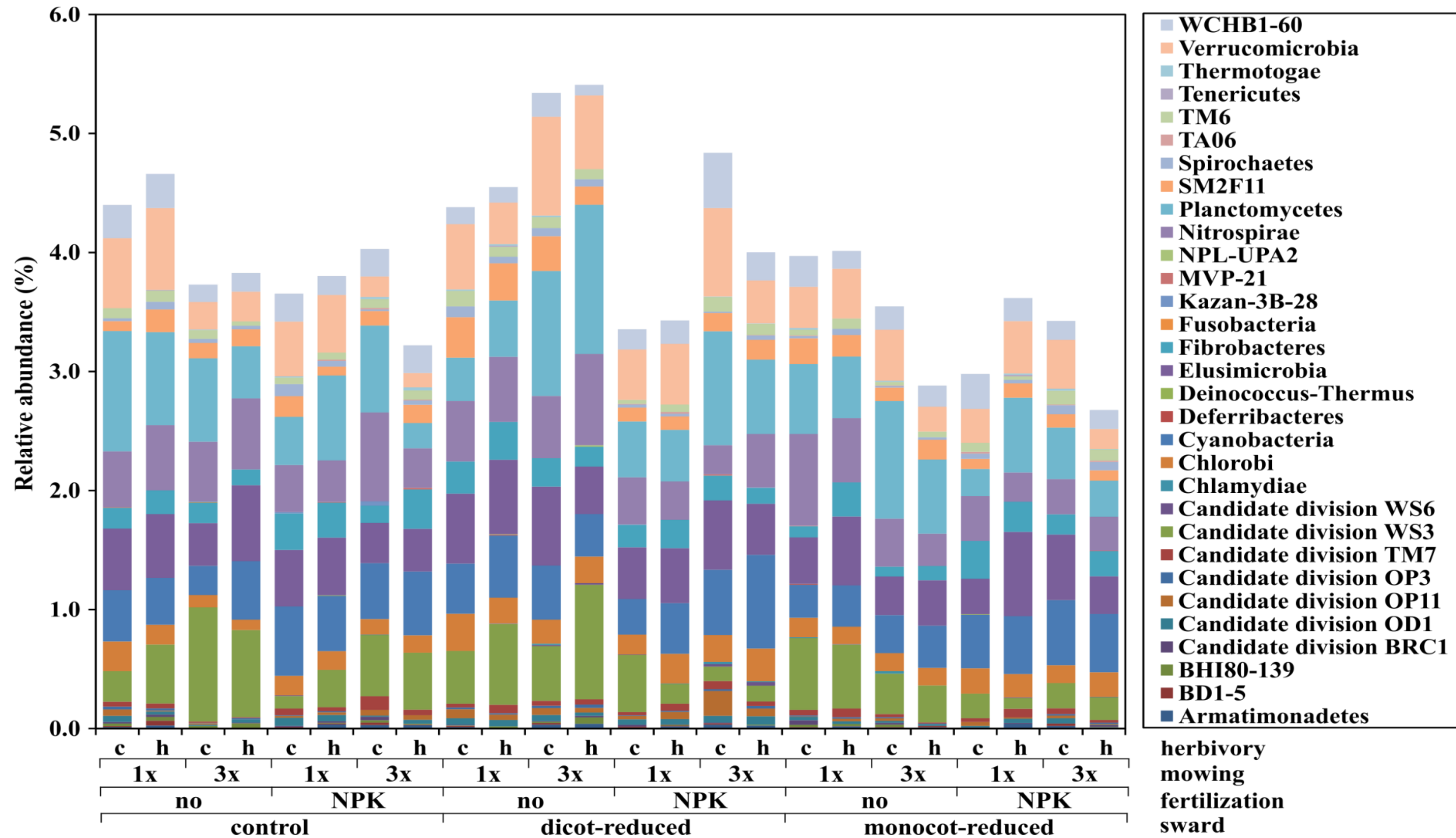

Fig. 2. Relative abundances of rare bacterial phyla as revealed by pyrosequencing-based analysis of generated 16S rRNA gene amplicons. Abundances are mean values of the three replications per treatment. Bacterial phyla with less than 1% mean abundance are shown.

In this study, 44,452 OTUs at 3% genetic divergence were detected in all samples. We identified 148 abundant bacterial OTUs at 3% genetic divergence (>0.1% of all classified sequences) (relative abundances of the 25 most abundant OTUs are shown in Supplemental Tab. S5). Together, these OTUs contributed for approximately 54.78% of the total bacterial community. The most abundant phylotype at a genetic distance of 3% across all samples was a *Bradyrhizobium*, belonging to the order *Rhizobiales*, representing 4.8% of all sequences. The second and third most abundant phylotypes at the same genetic distance were an uncultured *Acidobacterium* (unknown order) and the bacterium Ellin6561 (order *Rhizobiales*), representing 1.95 or 1.90% of the sequences, respectively.

In addition, sequences were related to several uncultured bacteria of the *Bacillaceae* (unknown order), *Nitrosomonadaceae* (order *Nitrosomonadales*), *Rhodospirillaceae* (order *Rhodospirales*), as well as an uncultured *Acidobacteria* bacterium (order Incertae Sedis, and *Catellatospora* sp., belonging to the order *Micromonosporales*. The 25 most abundant phylotypes and their taxonomic affiliations are shown in Tab. 3.

Sward composition-dependent bacterial communities

To investigate the impact of sward composition on bacterial richness, rarefaction curves and alpha diversity indices were calculated with regard to the three sward types. The rarefaction analysis revealed a significant decrease in bacterial richness at 80%, 97% and, 99% genetic distance in the herbicide-treated plots compared to the species-rich control plots (Fig. 3).

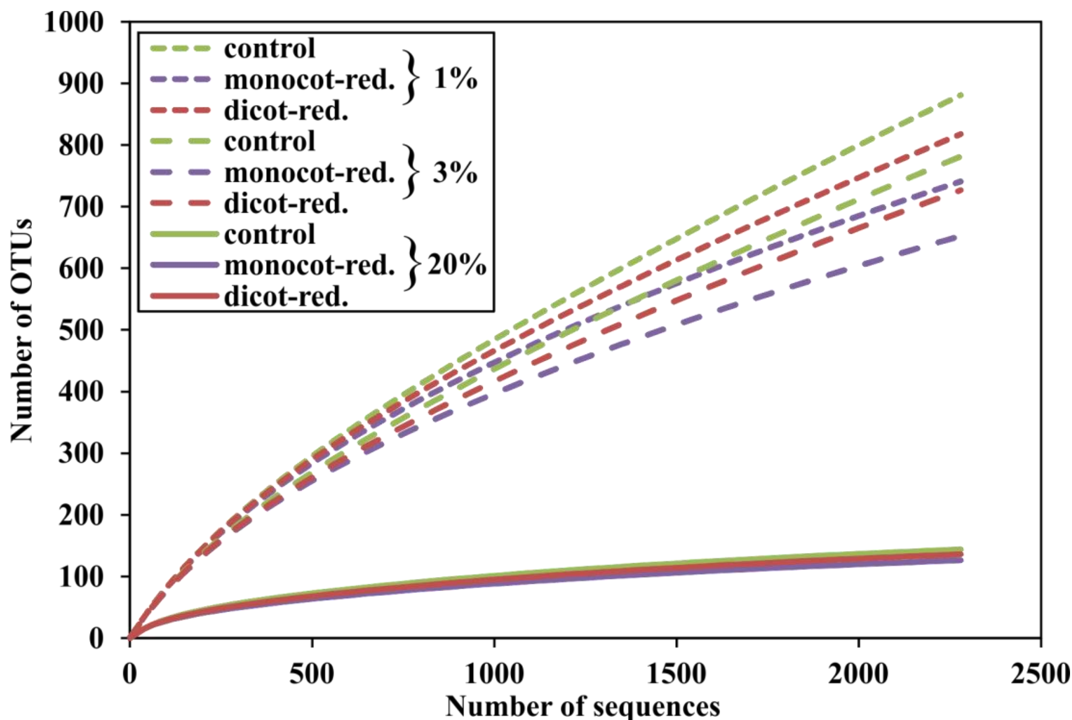

Fig. 3. Rarefaction curves at 99%, 97%, and 80% genetic distance with respect to sward diversity. Curves were calculated with QIIME (Caporaso *et al.*, 2010).

The lowest richness was recorded on the dicot-reduced plots at all three genetic distance levels (Tab. 1). The observed number of OTUs varied between 143.8±9.8 (control plots), 126.5±17.8 (dicot-reduced plots) and between 135.4±8.2 (monocot-reduced plots). The lower diversity in herbicide-treated plots was supported by the calculated alpha diversity indices. The Shannon index showed higher values at control plots (3.28 ±0.15) compared to dicot-reduced plots (3.14 ±0.19) and monocot-reduced plots (3.18 ±0.14) at genetic distances of 20%. The same results were obtained for genetic distances at 3 and 1%. The observed number of OTUs as well as the diversity indices at all three genetic distance levels were significantly (p value < 0.05) reduced in herbicide-treated plots. As a consequence, the decrease of plant species diversity also led to a reduction of bacterial diversity in the rhizosphere.

Tab. 1: Impact of sward diversity, fertilization, different mowing frequencies, and above-ground herbivory on bacterial richness at 99%, 97%, and 80% genetic distance. Alpha diversity indices were calculated with QIIME (Caporaso *et al*., 2010).

	Observed number of OTUs			*Maximal number of OTUs*			*ACE*			*Chao1*			*Shannon (H')*		
Sward type	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***
Control	143.8±9.8	779.6±97.2	883.4±114.4	188.2 ± 12.2	1747.7 ± 515.3	2146.0 ± 761.7	223.8 ± 16.0	2985 ± 1042	3980 ± 2005	221.6 ± 19.6	3159 ± 806.2	5044 ± 2030	3.28 ± 0.15	5.77 ± 0.15	6.00 ± 0.15
Dicot-reduced	126.5±17.8	650.9±111.3	743.8±133.3	164.7 ± 26.5	1179.0 ± 402.9	1404.6 ± 524.7	211.8 ± 10.8	2457 ± 897.1	2998 ± 1667	211.2 ± 13.9	2762 ± 830.2	4031 ± 2137	3.14 ± 0.19	5.63 ± 0.21	5.87 ± 0.22
Monocot-reduced	135.4±8.2	726.7±76.5	821.0±90.9	177.7 ± 11.1	1493.7 ± 384.6	1765.2 ± 581.0	203.4 ± 31.2	1641 ± 795.4	1880 ± 1045	197.6 ± 29.8	1812 ± 852.8	2256 ± 1359	3.18 ± 0.14	5.70 ± 0.15	5.95 ± 0.15
Fertilization	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***
Control	135.6 ± 15.8	708.7 ± 108.1	799.6 ± 133.7	176.8 ± 22.9	1420.9 ± 443.8	1693.8 ± 624.1	214 ± 25	2243 ± 946	2740 ± 1546	211 ± 26	2491 ± 932	3522 ± 1948	3.23 ± 0.16	5.69 ± 0.15	5.91 ± 0.17
Fertilized	134.9 ± 13.1	729.5 ± 110.2	832.5 ± 119.0	176.9 ± 17.6	1525.9 ± 538.2	1850.0 ± 759.5	212 ± 20	2479 ± 1171	3165 ± 2063	209 ± 23	2665 ± 1064	4032 ± 2398	3.17 ± 0.17	5.71 ± 0.21	5.97 ± 0.19
Mowing frequency	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***
One	131.9 ± 13.6	697.0 ± 94.5	790.2 ± 119.3	172.0 ± 19.9	1364.1 ± 389.5	1626.7 ± 540.0	208 ± 21	2143 ± 905	2614 ± 1446	205 ± 21.8	2354 ± 889	3440 ± 1990	3.18 ± 0.15	5.69 ± 0.14	5.91 ± 0.17
Thrice	138.6 ± 14.6	741.2 ± 118.9	841.9 ± 130.4	181.7 ± 19.8	1582.8 ± 562.6	1917.1 ± 803.1	218 ± 23	2579 ± 1175	3291 ± 2102	216 ± 25.1	2802 ± 1061	4113 ± 2343	3.22 ± 0.19	5.71 ± 0.21	5.97 ± 0.20
Herbivory	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***	***80%***	***97%***	***99%***
Control	135.3 ± 12.2	719.7 ± 93.9	814.9 ± 110.7	177.4 ± 17.6	1458.7 ± 417.5	1728.9 ± 567.9	215.1 ± 19.1	2331.8 ± 988.4	2842.4 ± 1586.4	212.4 ± 21.5	2557.9 ± 966.1	3733.9 ± 2194.8	3.20 ± 0.14	5.71 ± 0.14	5.95 ± 0.15
Herbivory	135.1 ± 16.4	718.5 ± 123.4	817.2 ± 142.5	176.3 ± 22.9	1488.2 ± 563.4	1815.0 ± 807.7	210.8 ± 25.8	2390.2 ± 1146.9	3062.6 ± 2048.0	207.8 ± 26.4	2598.0 ± 1040.3	3819.7 ± 2203.4	3.20 ± 0.20	5.69 ± 0.22	5.93 ± 0.21

The PCoA analysis revealed that species-rich control plots shared a more similar community structure followed by monocot-reduced plots. Dicot-reduced plots exhibited a more dissimilar community structure when compared to the other sward types (Fig. 4).

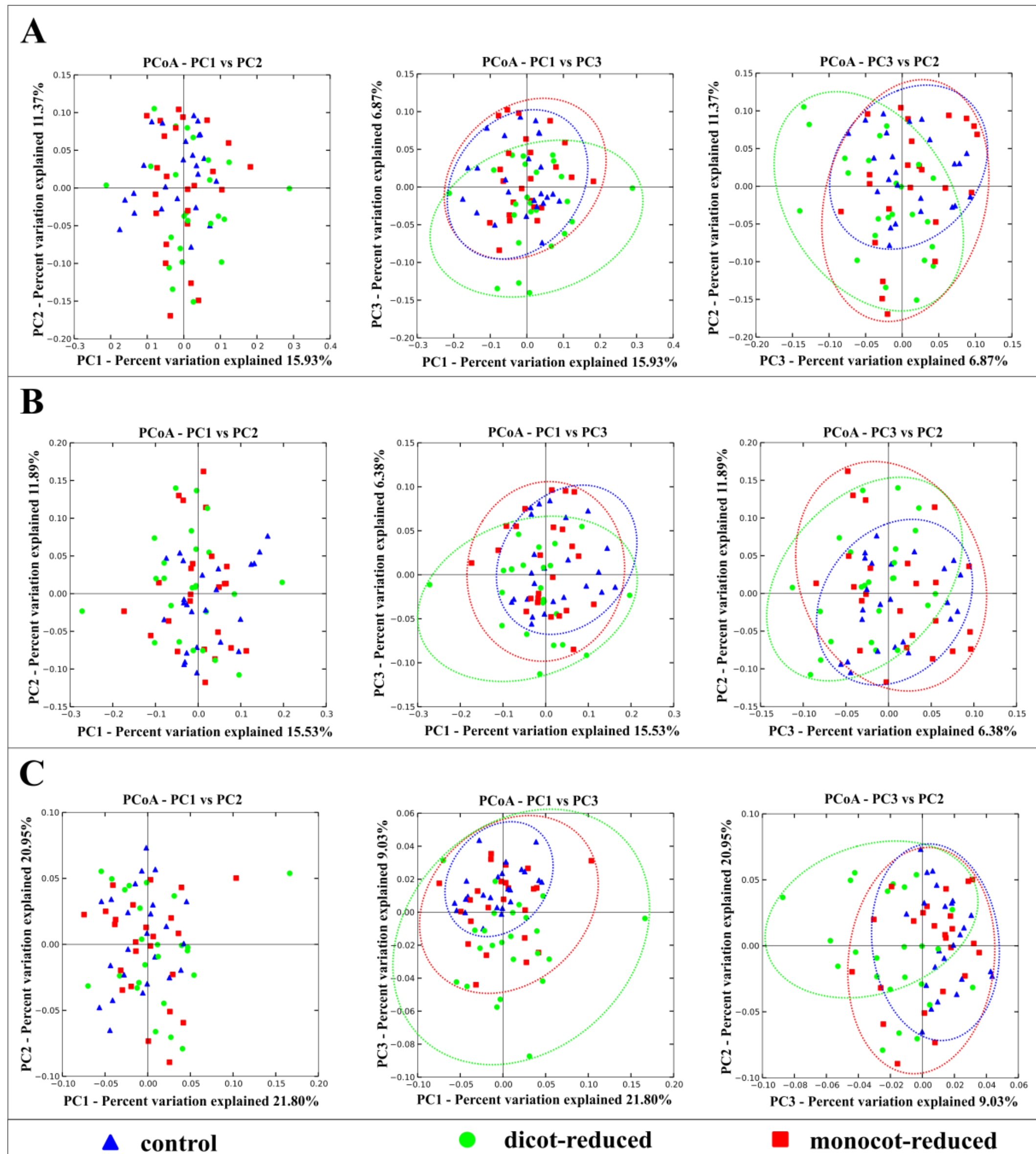

Fig. 4: Impact of sward composition on bacterial community structures in the rhizosphere at 99% (A), 97% (B), and 80% (C). PCoA plots were calculated with QIIME (Caporaso *et al.*, 2010). Circles are drawn to highlight differences between the different sward types.

Bacterial community composition is affected by fertilization and different mowing frequencies

Bacterial community composition in regard to different management regimes was initially assessed by DGGE analysis of partial 16S rRNA gene sequences. DGGE of species-rich plots revealed complex patterns with approximately 20 bands for each treatment (Fig. 5). The same results were obtained for monocot-reduced plots (Supplemental Fig. S2), while DGGE of dicot-reduced plots revealed complex patterns with more than 30 bands (Supplemental Fig. S3).

Cluster analysis of DGGE was performed with regard to different grassland management regimes (with vs. without NPK fertilization; mowing once vs. thrice per year), and above-ground herbivory for the three different sward compositions. UPGMA dendrograms of bacterial communities in the plant rhizosphere showed that the different management regimes and herbivory influenced the composition of bacterial communities. For example, cluster analysis of the DGGE patterns of the rhizosphere bacterial community of species-rich plots revealed a strong impact of fertilizer application on community composition (Fig. 6A).

The effect of mowing frequency was influenced by the fertilization regime. Samples derived from unfertilized plots exhibited distinct cluster formation for the two mowing frequencies, indicating a more similar community composition in the once and thrice mown plots, respectively. However, some samples collected from the fertilized plots mown thrice as well as from the fertilized plots mown once grouped also in distinct clusters. In contrast to these findings, the above-ground herbivory did not strongly impact the bacterial community in the rhizosphere, although some samples exhibited distinct clusters. Similar results were observed for the bacterial community composition in the plant rhizosphere in samples collected from dicot-reduced (Fig. 6B) as well as from monocot-reduced plots (Fig. 6C).

To gain a more detailed picture about the changes of bacterial community in the rhizosphere in response to management regimes and above-ground herbivory, we analyzed the samples with next generation sequencing.

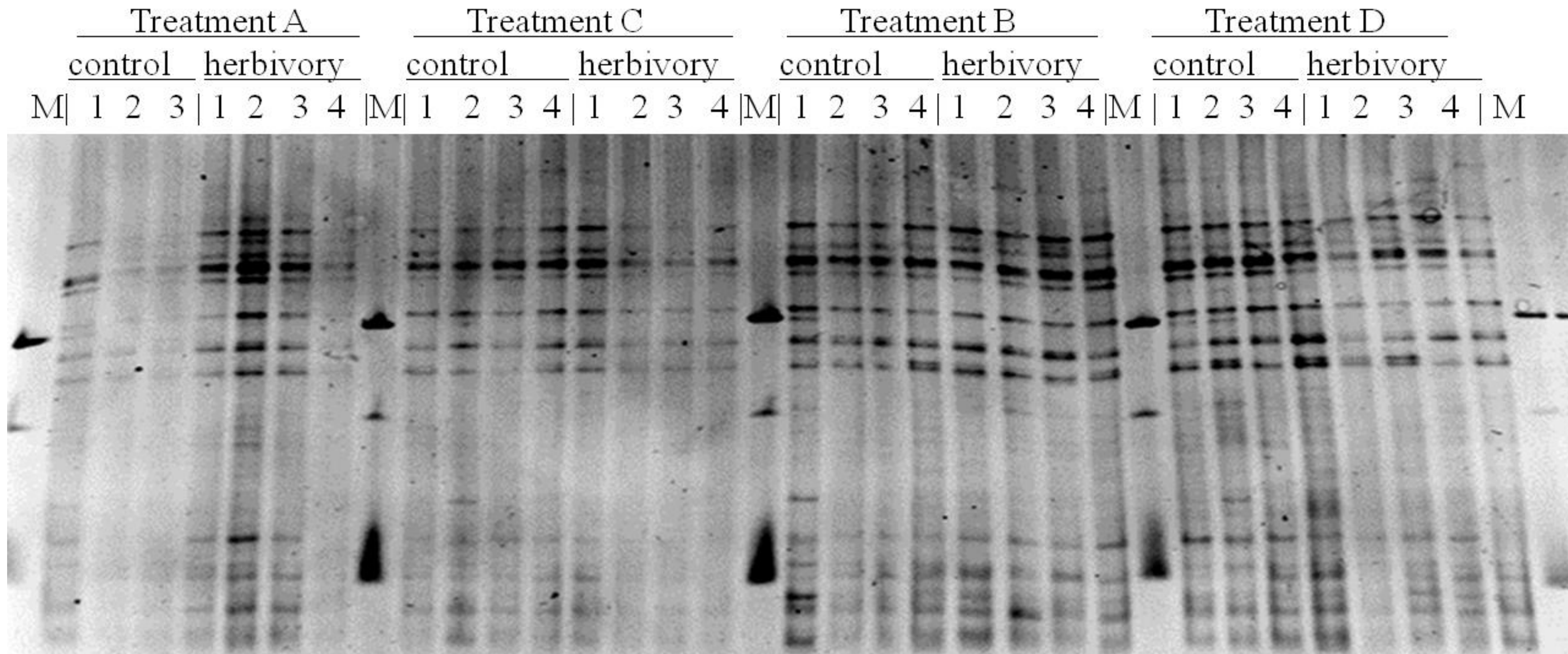

Fig. 5. DGGE profile of species-rich plots showing the influence of different fertilization and mowing regimes as well as above-ground herbivory on bacterial endophyte communities in the rhizosphere. Soil samples were taken in summer 2011. Independent replicates are indicated with numbers from 1 to 4. Treatment A: 1 x mowing/ year, no NPK; treatment B: 3 x mowing/ year, no NPK; treatment C: 1 x mowing/ year, NPK; treatment D: 3 x mowing/ year, NPK. M: GeneRuler 1 kb DNA Ladder (Fermentas, St. Leon-Rot, Germany).

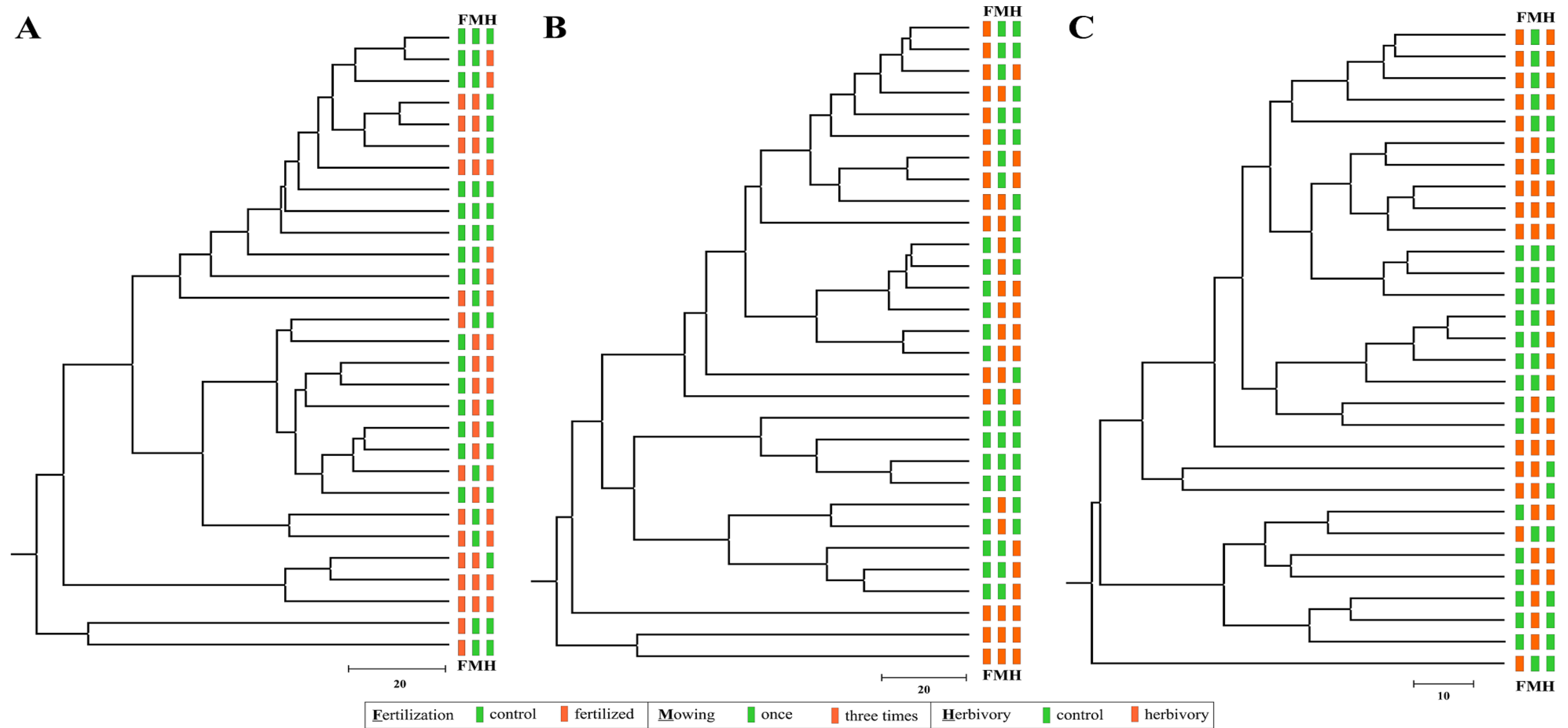

Fig. 6. UPGMA dendrogram generated by cluster analysis of DGGE fingerprints on the influence of different management regimes and above-ground herbivory on the bacterial community in the rhizosphere for (A) species-rich plots, (B) dicot-reduced plots, and (C) monocot-reduced plots. Soil samples were taken in summer 2011. The dendrogram was constructed using the Pearson correlation coefficient. The scale shows similarity values.

To investigate the impact of fertilization application and mowing frequencies on bacterial richness, rarefaction curves and alpha diversity indices were calculated with regard to these regimes. The rarefaction analysis revealed an increase in bacterial richness at 97% and 99% genetic distance in the fertilized plots compared to the control plots (Fig. 7A). Despite the recorded change, this increase in richness was not supported by the calculated alpha diversity indices (Tab. 1). The observed number of OTUs as well as the diversity indices at all three genetic distance levels did not significantly (p value < 0.05) differ in the fertilized and unfertilized plots. As a consequence, the fertilizer application did not significantly affect the bacterial richness in the rhizosphere.

A comparison of rarefaction curves with regard to the two mowing frequencies revealed a higher bacterial richness at all three genetic distance levels in the plot mown three times compared to the plots mown only once (Fig. 7B). The observed number of OTUs at all three genetic distance levels were significantly (p value < 0.05) higher in the plots mown three times (138.6±14.6, 741.2±118.9, 841.9±130.4 compared to 131.9±13.6, 697.0±94.5, 790.2±119.3 in once mown plots at a genetic distance of 20, 3, and 1%, respectively). The same was recorded for the maximal number of OTUs. Thus, an increasing number of mowing events led to an increase of bacterial richness in the rhizosphere.

This higher richness was supported by the calculated alpha diversity indices (Tab. 1). ACE and Chao1 indices were significantly higher at 97% and 80% genetic distance only. In contrast to this, no differences was recorded for the calculated Shannon indices (3.18±0.15, 5.69±0.14, 5.91±0.17 in thrice mown plots compared to 3.22±0.19, 5.71±0.21, 5.97±0.20 in once mown plots at a genetic distance of 20, 3, and 1%, respectively).

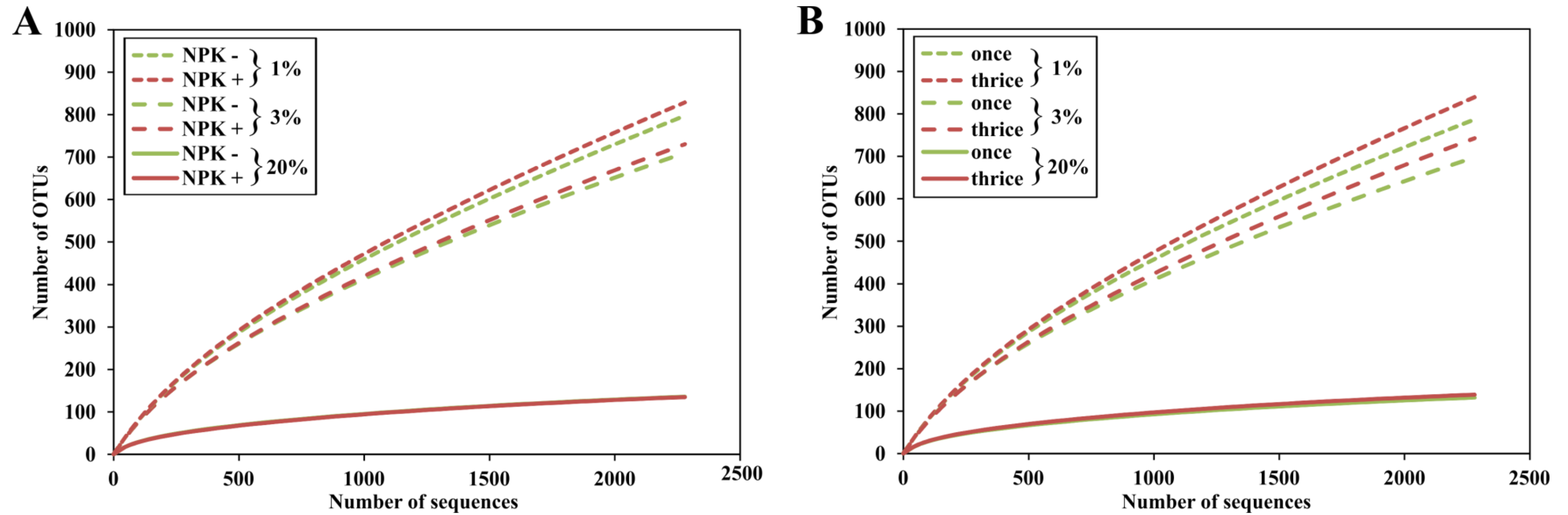

Fig. 7. Rarefaction curves at 99%, 97%, and 80% genetic distance with respect to fertilizer application (A) and mowing frequencies (B). Curves were calculated with QIIME (Caporaso *et al.*, 2010).

We further validated the impact of fertilization as well as mowing frequency on bacterial community structures by Principal Coordinate Analysis (PCoA).Whereas no difference in the generated PCoA plots was found at 80% genetic distance, plots exhibited a clear separation between fertilized and control plots at 97% and 99% genetic dissimilarity indicating a strong influence of fertilizer application on bacterial community structures (Fig. 8). As calculated plots did not show separation or cluster formation of differently treated plots, mowing frequencies (Fig. 9) did not impact bacterial community structure.

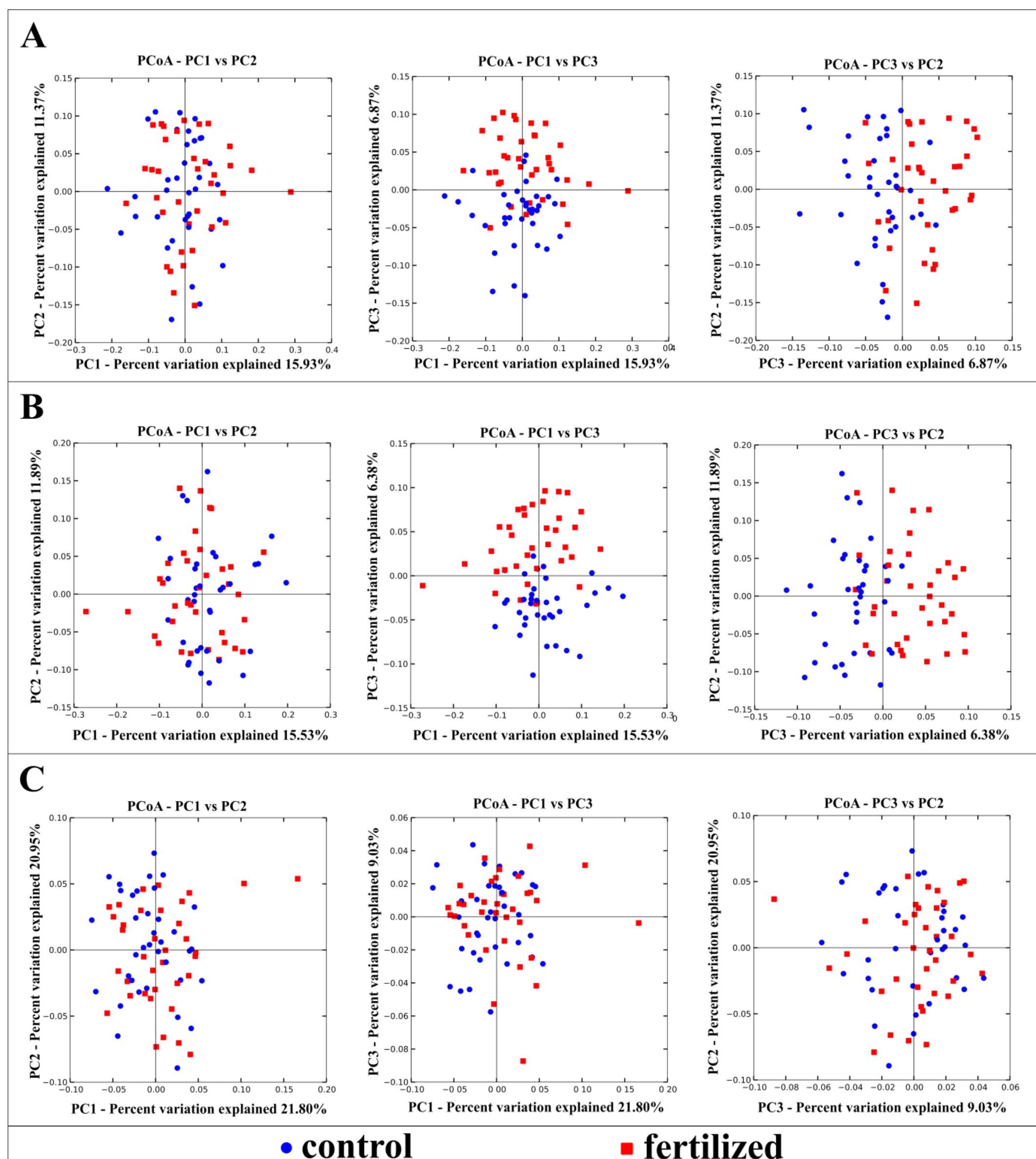

Fig. 8. Impact of fertilization on bacterial community structures at 99% (A), 97% (B), and 80% (C). PCoA plots were calculated with QIIME (Caporaso *et al.*, 2010).

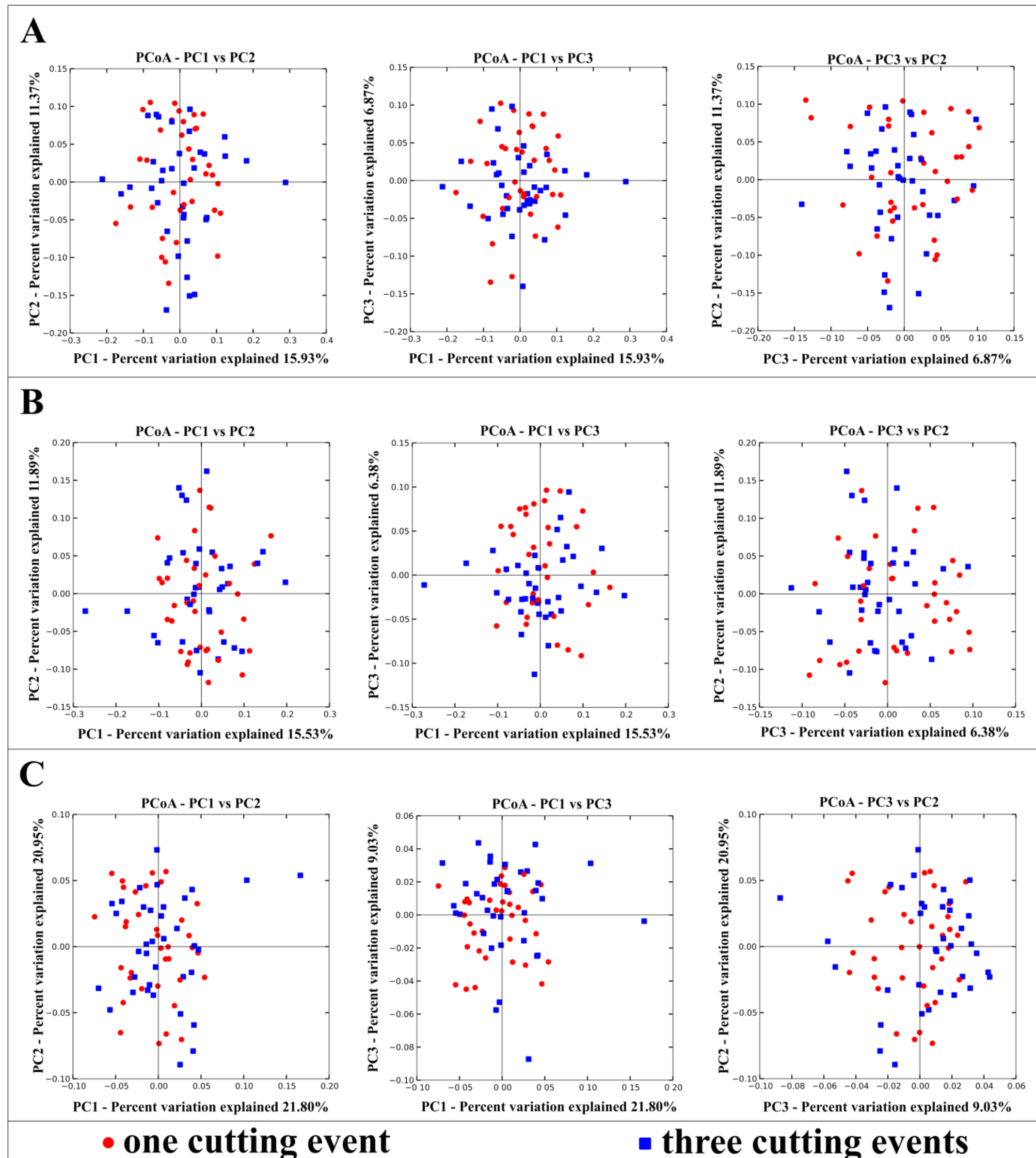

Fig. 9. Impact of mowing frequencies on bacterial community structures at 99% (A), 97% (B), and 80% (C). PCoA plots were calculated with QIIME (Caporaso *et al.*, 2010).

Changes in bacterial community composition with regard to herbivory

A comparison of rarefaction curves and alpha diversity indices with regard to the herbivory treatments did not reveal any differences between control and herbivory plots (Fig. 10). The observed number of OTUs as well as the diversity indices at all three genetic distance levels did not significantly (p value < 0.05) differ in the control and herbivory plots (Tab. 1).

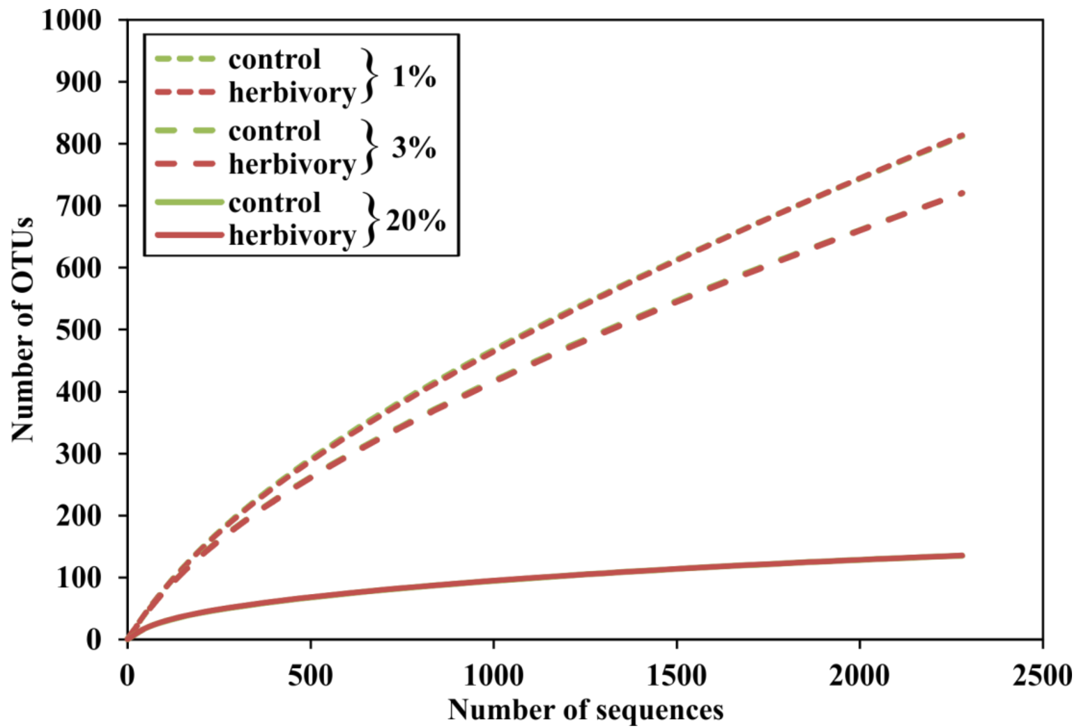

Fig. 10. Rarefaction curves at 99%, 97%, and 80% genetic distance with respect to herbivory. Curves were calculated with QIIME (Caporaso *et al.*, 2010).

We further validated the impact of the above-ground herbivory on bacterial community structures by Principal Coordinate Analysis (PCoA) (Fig. 11). No differences in the generated PCoA plots were found at the three genetic distances. Thus, herbivory had no impact on the bacterial community structure.

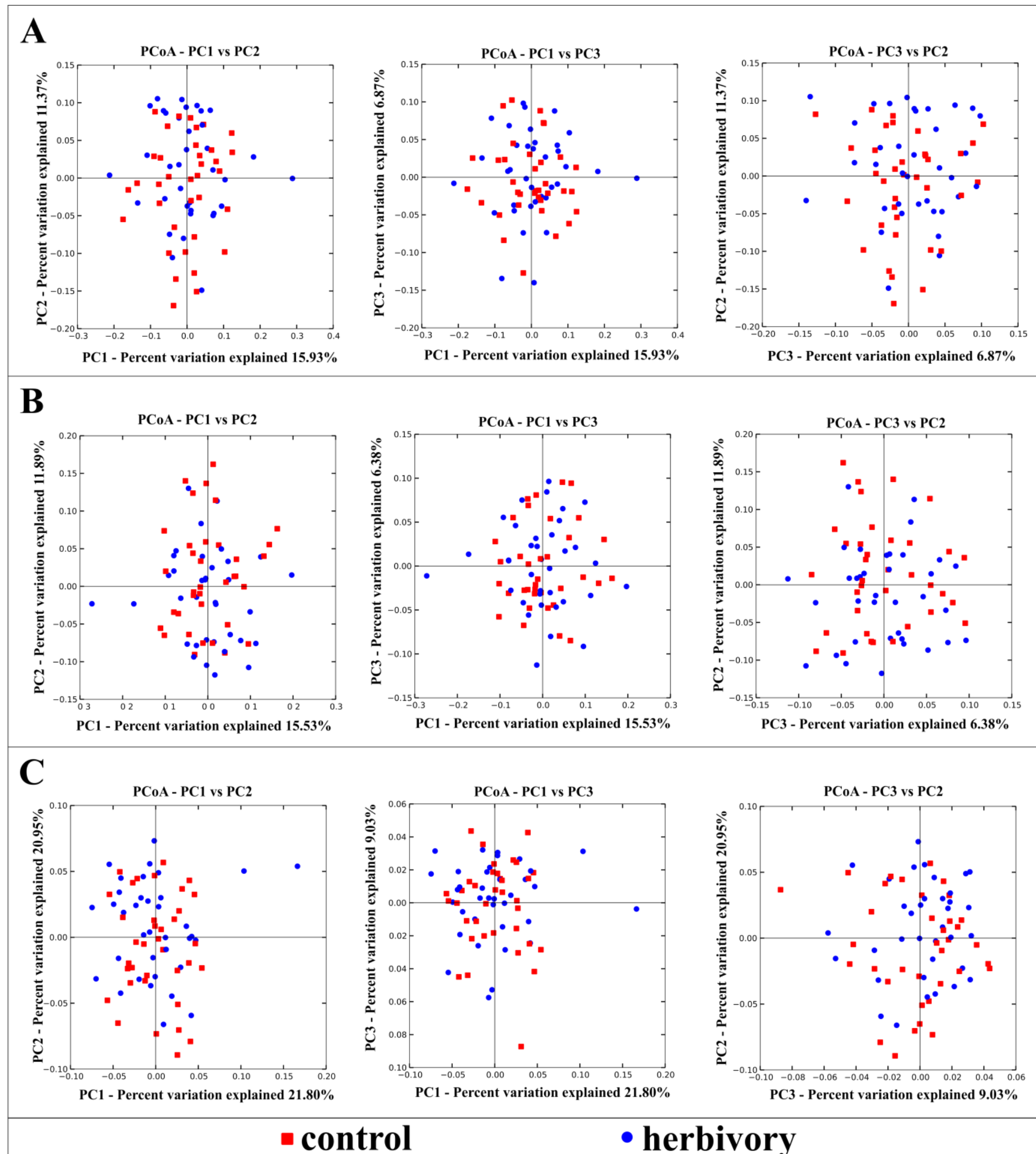

Fig. 11. Impact of above-ground herbivory on bacterial community structures at 99% (A), 97% (B), and 80% (C). PCoA plots were calculated with QIIME (Caporaso *et al.*, 2010).

Sward diversity, management regimes, and above-ground herbivory alter the bacterial community in the rhizosphere

We analyzed the effect of management regimes, sward composition, and above-ground herbivory on the relative abundance of predominant bacterial groups and species by statistical modeling using Dirichlet regression. The sward composition had a significant influence (p value < 0.05) on the *Firmicutes* and the *Gamma-proteobacteria* (Tab. 2).

Tab. 2: Effect of different fertilization regimes, mowing frequencies, herbicide application, above-ground herbivory, and the combination of these treatments on bacterial phyla and proteobacterial classes.

phylum or proteobacterial class	*dicot-reduced*	*monocot-reduced*	*fertilization*	*mowing*	*herbivory*	*dicot-reduced:fertilization*	*monocot-reduced:fertilization*	*dicot-reduced:mowing*	*monocot-reduced:mowing*	*fertilization:mowing*	*dicot-reduced:herbivory*	*monocot-reduced:herbivory*	*fertilization:herbivory*	*Mowing:herbivory*	*dicot-reduced:fertilization:mowing*	*monocot-reduced:fertilization:mowing*	*dicot-reduced:fertilization:herbivory*	*monocot-reduced:fertilization:herbivory*	*dicot-reduced:mowing:herbivory*	*monocot-reduced:mowing:herbivory*	*fertilization:mowing:herbivory*	*dicot-reduced:fertilization:mowing:herbivory*	*monocot-reduced:fertilization:mowing:herbivory*
Acidobacteria			***	***		***	***	**	*	***			*		***	***	*	**			***	**	***
Actinobacteria			***	***		*	**	*	.	***			.	**	*	***	.	*			***	***	***
Bacteroidetes			**	**		**	.		.	***				*	**	**	.				**	***	*
Chloroflexi			**	***		.	*	.	.	***				*	.	**					**	**	**
Cyanobacteria			*			*				*						.							
Elusimicrobia			*	.		.	.			**					.	**							*
Fibrobacteres			*			.				**						.					*		
Firmicutes	*	*	*	***			*	**	*	***		*			**	**		*	.		*	***	***
Gemmatimonadetes			***	***		*	*	.	*	***					*	***		*			**	*	***
Nitrospirae			.	*			*			*													
Planctomycetes										.													
Alphaproteobacteria			***	***		**	**	*		***			*	.	**	***	*	*			***	***	***
Betaproteobacteria			***	***		**	***	*	**	***			.	*	*	***	*	*			**	**	***
Gammaproteobacteria	*		***	***		***	**	**	*	***			*	*	***	***	**	*			***	***	***
Deltaproteobacteria			***	***		**	**		*	***			.		*	***	.	*			**	**	***
Verrucomicrobia			.							**						.						.	
WS3				***				*	**	*						*							

*** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05; . p-value < 0.1

The abundance of *Firmicutes* was significantly enhanced on all herbicide-treated plots (Fig. 12A), while the *Gammaproteobacteria* did only respond to the herbicide application targeting dicots (Tab. 2). In combination with the other investigated parameters, sward composition affected almost all bacterial phyla and proteobacterial classes such as the *Nitrospirae*. This phylum was significantly influenced by fertilization on the monocot-reduced plots (Tab. 2).

We further analyzed the impact of sward composition on the relative abundance of predominant bacterial phylotypes (Supplemental Tab. S5). The results for the top 25 OTUs (3% genetic distances) are shown in Tab. 3. The abundance of several of the analyzed OTUs was affected by at least one parameter. Sward composition, fertilizer application and mowing frequency had the highest impact on bacterial abundance. Many bacterial phylotypes were influenced by herbicide treatment against dicots and/or monocots. Whereas the bacterium Ellin6561 (order *Rhizobiales*) and some uncultured bacteria of the orders *Acidobacteriales*, *Rhodospirillales*, and *Rhizobiales* were significantly affected by herbicide application against dicots, some uncultured bacterium of the *Bacillaceae* (unknown order) and of the order *Frankiales*, as well as an uncultured *Acidobacterium* (unknown order) were influenced by herbicide application against monocots.

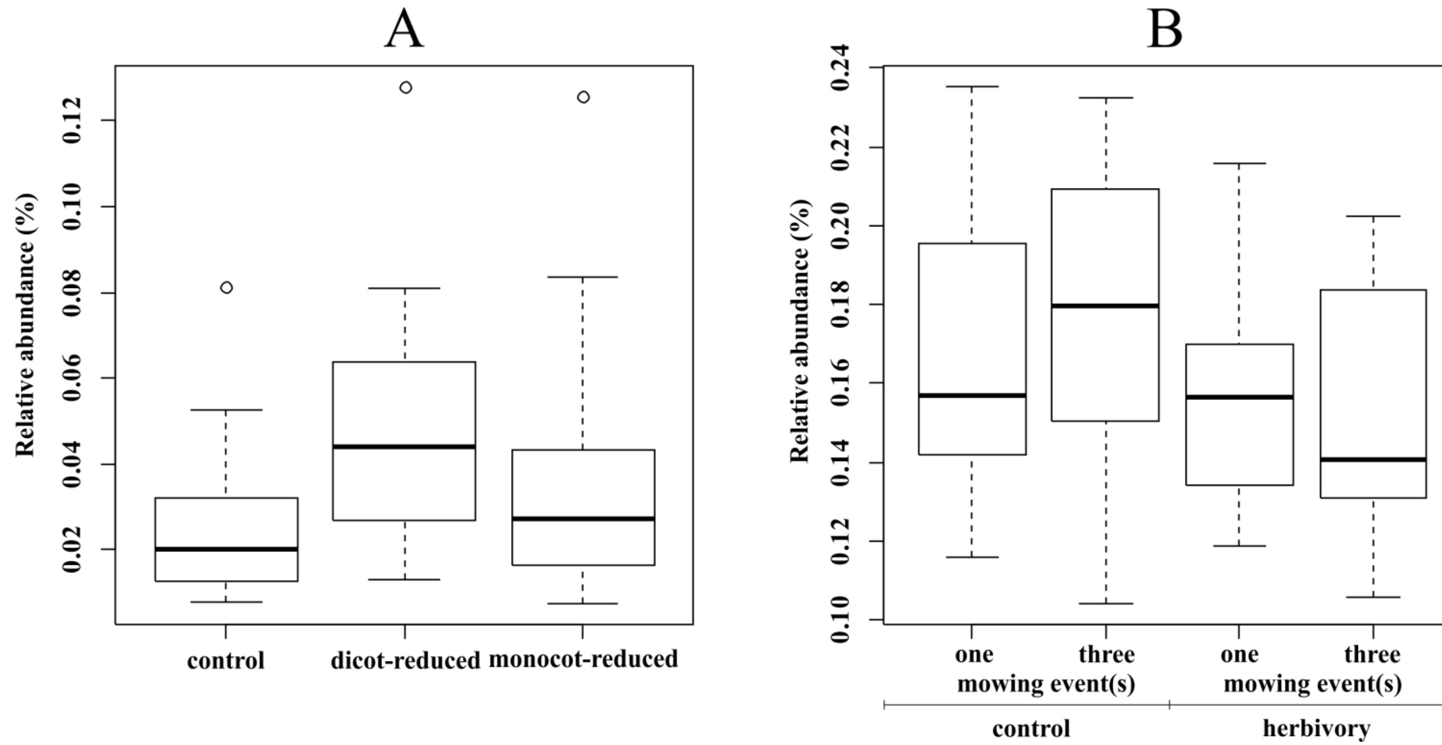

Fig. 12. Effect of sward composition on the abundance of *Firmicutes* (A) and of mowing frequency in combination with herbivory on the abundance of *Actinobacteria* (B).

Tab. 3: Effect of different fertilization regimes, mowing frequencies, herbicide application, above-ground herbivory, and the combination of these treatments on the 25 most abundant bacterial OTUs (3% genetic divergence).

OTU ID	*dicote-reduced*	*monocots-reduced*	*fertilization*	*mowing*	*herbivory*	*dicote-reduced:fertilization*	*monocots-reduced:fertilization*	*dicote-reduced:mowing*	*monocots-reduced:mowing*	*fertilization:mowing*	*dicote-reduced:herbivory*	*monocots-reduced:herbivory*	*fertilization:herbivory*	*Mowing:herbivory*	*dicote-reduced:fertilization:mowing*	*monocots-reduced:fertilization:mowing*	*dicote-reduced:fertilization:herbivory*	*monocots-reduced:fertilization:herbivory*	*dicote-reduced:mowing:herbivory*	*monocots-reduced:mowing:herbivory*	*fertilization:mowing:herbivory*	*dicote-reduced:fertilization:mowing:herbivory*	*monocots-reduced:fertilization:mowing:herbivory*	*taxonomic affiliation*
15254							*			*							**							Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Bradyrhizobiaceae.Bradyrhizobium.Bradyrhizobium.sp.
3020	**	***	**	*		*	***		*	**						**		*	**					Bacteria.Acidobacteria.Acidobacteria.Candidatus.Solibacter.uncultured.Acidobacteria.bacterium
15334	***			*	*			***		*		**					*	*					**	Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Xanthobacteraceae.uncultured.bacterium.Ellin6561
430			**			*				*							**		**					Bacteria.Acidobacteria.Acidobacteria.Acidobacteriales.Acidobacteriaceae.Candidatus.Koribacter.uncultured.bacterium
31887		**				*			*		*	***					*		***	*		**		Bacteria.Firmicutes.Bacilli.4.15.uncultured.Bacillaceae.bacterium
10498				*			*	**	*								*	*	*					Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.alphaI.cluster.uncultured.bacterium
33544																			*					Bacteria.Acidobacteria.Acidobacteria.DA052.uncultured.bacterium
30880			***			***	*			**			**		*		**				*			Bacteria.Acidobacteria.Acidobacteria.Order.Incertae.Sedis.Family.Incertae.Sedis.Bryobacter.uncultured.Acidobacteria.bacterium
26950	***	**	***			***	***								**		**	*	**					Bacteria.Acidobacteria.Acidobacteria.Acidobacteriales.Acidobacteriaceae.uncultured.uncultured.bacterium
15204			***		*	***	***			***			**	*	***	***	**	***			***	*	***	Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Family.Incertae.Sedis.Rhizomicrobium.uncultured.bacterium
35896	*	*		***				*	**							**								Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Comamonadaceae.uncultured.uncultured.bacterium
1434			*				*	*				*	*				*	**	**					Bacteria.Bacteroidetes.Sphingobacteriia.Sphingobacteriales.Chitinophagaceae.uncultured.uncultured.bacterium
43557	**	**	***	***		**	**	***	***	***			**	***	***	***	**			***	**	*	***	Bacteria.Acidobacteria.Acidobacteria.Acidobacteriales.Acidobacteriaceae.uncultured.uncultured.bacterium
10655	**	*							*		*													Bacteria.Bacteroidetes.Cytophagia.Cytophagales.Cytophagaceae.Flexibacter.uncultured.bacterium
42418		*			**		*	*	***		**	***		*	*	***		***	***	***			***	Bacteria.Actinobacteria.Actinobacteria.Frankiales.Acidothermaceae.Acidothermus.uncultured.bacterium
10041	***		*			***		***			**				***		*		*					Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Beijerinckiaceae.uncultured.uncultured.proteobacterium
23893			**				*			***			*			**	*	*			***	**	***	Bacteria.Proteobacteria.Alphaproteobacteria.Rhodospirillales.Acetobacteraceae.uncultured.uncultured.bacterium
24136	*	**		***	*		*	*	*	*					*									Bacteria.Proteobacteria.Alphaproteobacteria.Rhizobiales.Xanthobacteraceae.uncultured.uncultured.bacterium
17761	*					**	*							*	**		*		**					Bacteria.Acidobacteria.Acidobacteria.Acidobacteriales.Acidobacteriaceae.uncultured.uncultured.bacterium
10097		**		*			*	***			*		*				***				*	*	*	Bacteria.Acidobacteria.Acidobacteria.Candidatus.Solibacter.uncultured.Acidobacteria.bacterium
1655	*								*					*		*	*		*	*			*	Bacteria.Proteobacteria.Alphaproteobacteria.Rhodospirillales.DA111.uncultured.bacterium
30883			**	*		**				*	**		*				***				*	**		Bacteria.Proteobacteria.Betaproteobacteria.Nitrosomonadales.Nitrosomonadaceae.uncultured.uncultured.beta.proteobacterium
41226	*		*	**						*			*				*					*	*	Bacteria.Proteobacteria.Alphaproteobacteria.Rhodospirillales.DA111.uncultured.bacterium
22924										**											*			Bacteria.Proteobacteria.Alphaproteobacteria.Rhodospirillales.JG37.AG.20.uncultured.Rhodospirillaceae.bacterium
38931								**							**				**		*	***		Bacteria.Acidobacteria.Acidobacteria.Candidatus.Solibacter.uncultured.Acidobacteria.bacterium

*** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05.

In combination with the other investigated parameters, sward composition had a significant effect on almost all bacterial phylotypes of the top 25 OTUs. These impacts were stronger for the more abundant phylotypes than for rare phylotypes (Tab. 3, Supplemental Tab. S5). This is also true for fertilization, mowing frequency, and above-ground herbivory separately or in combination with each other (Supplemental Tab. S5).

The majority of the abundant bacterial phyla and proteobacterial classes was significantly affected by fertilizer application and different mowing frequencies (Tab. 2). *Acidobacteria* were significantly less abundant on fertilized plots (Fig. 13A). The opposite was recorded for *Actinobacteria* (Fig. 13B). When analyzing the effect of the parameters fertilization or mowing separately or in combination, we found synergistic effects. The abundance of the phylum *Bacteroidetes* was significantly affected to a higher extend by fertilization and mowing frequency as by fertilization or mowing separately (Fig. 13C). On the other hand, the relative abundance of this phylum was reduced by fertilization on plots mown once, but it increased by fertilization on plots mown thrice. The same effect was recorded for *Chloroflexi* (data not shown). Moreover, the abundance of *Verrucomicrobia* was significantly affected by fertilization and mowing but not by fertilization or mowing only (Tab. 2).

In addition, fertilization as well as mowing frequency and the combination of both treatments had a significant impact on most phylotypes of the top 25 OTUs (Tab. 3). The most abundant phylotype was affiliated to *Bradyrhizobium.* The abundance of this OTU was reduced by fertilization (Fig. 14A), but only significantly on plots mown thrice per year (Fig. 14B). In addition, the abundance was decreased by fertilization on monocot-reduced plots (Fig. 14C).

We did not find direct correlations between above-ground herbivory and the abundance of predominant bacterial groups. However, significant changes in combination with other treatments were detectable (Tab. 3). For example, the abundance of *Actinobacteria* was significantly reduced by herbivory, but only on plots mown three times per year (Fig. 12B). As only few OTUs reacted towards herbivory, its influence on community structure must be considered to be weaker as originally expected.

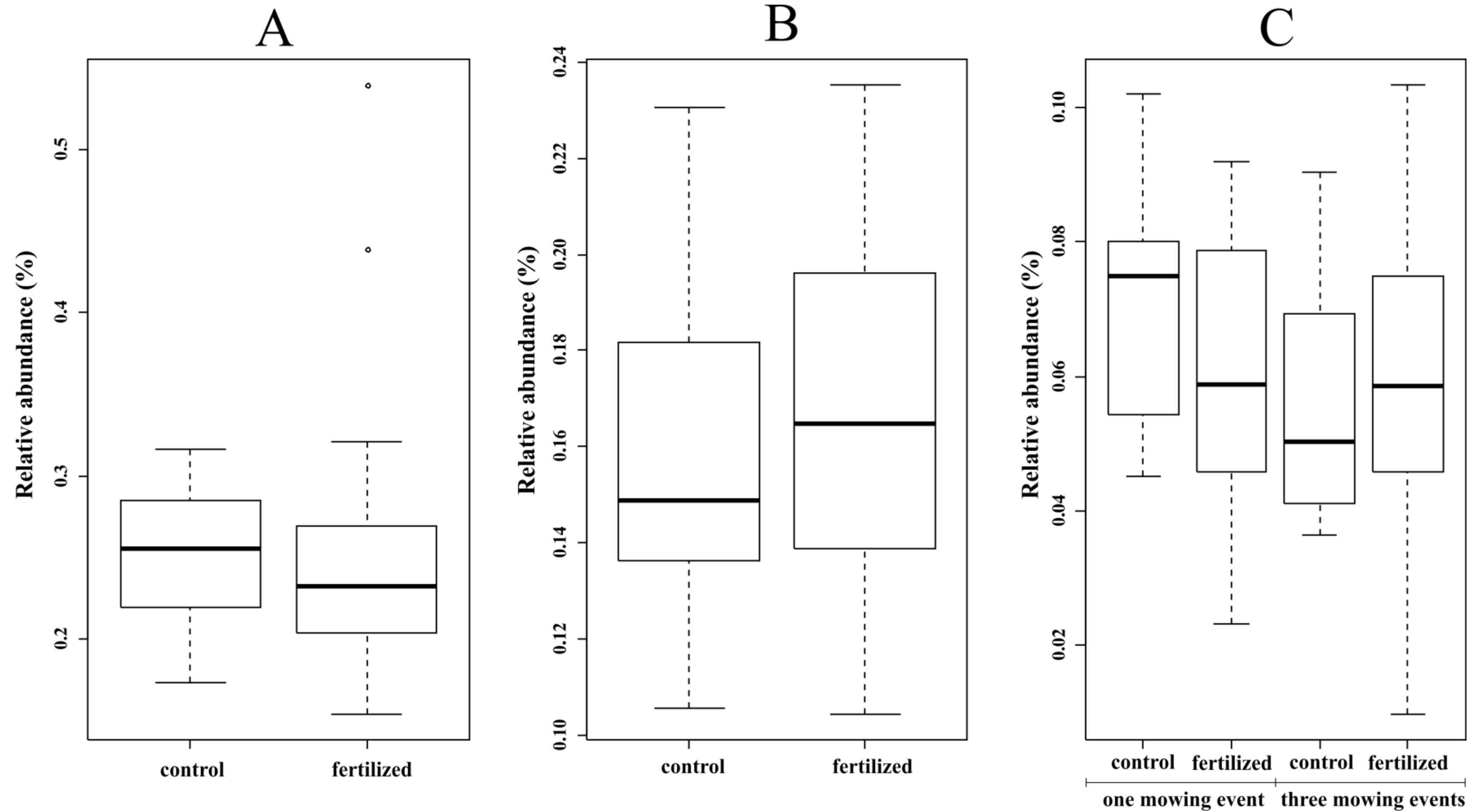

Fig. 13. Effect of fertilization on the *Acidobacteria* (A) and *Actinobacteria* (B) as well as the effect of fertilization and mowing regimes on *Bacteroidetes* (C).

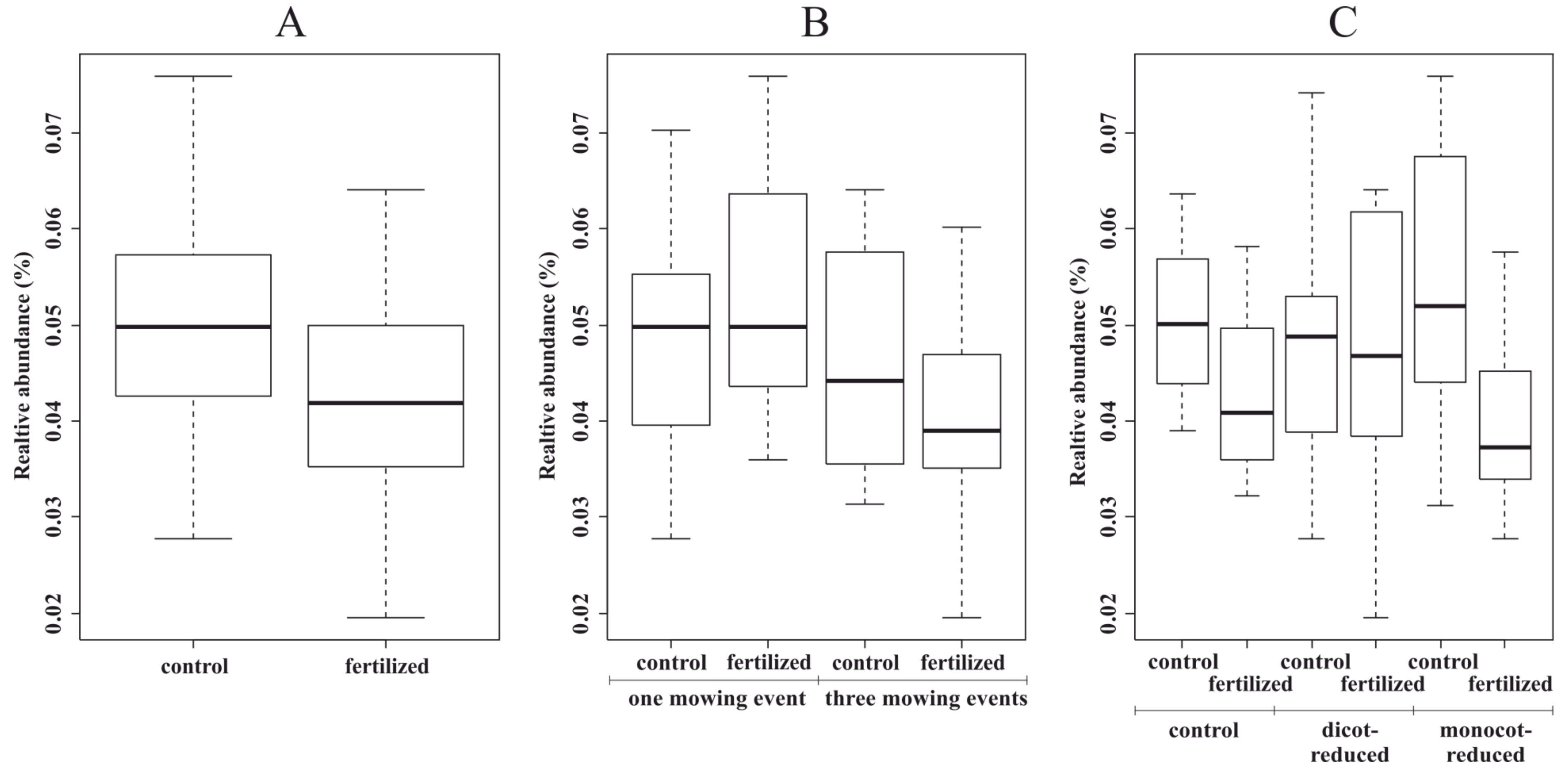

Fig. 14. Effect of fertilization (A), fertilization and mowing (B), and sward composition and fertilization (C) on the abundance of an OTU affiliated to *Bradyrhizobium* sp.

Discussion

Characterization of bacterial community structure in the rhizosphere

To gain insights into the bacterial community structures in the rhizosphere in the grassland system, we applied 454 pyrosequencing. The seven predominant bacterial phyla and the 4 proteobacterial classes observed in this study agreed with other studies (Gardner *et al.*, 2011, Nacke *et al.*, 2011). In this study, 44,452 OTUs at 3% genetic divergence were detected in all samples (Supplemental Tab. S5). Some of them are known as typical soil or rhizosphere bacteria such as *Bradyrhizobium* (order *Rhizobiales*), *Bacillus* (order *Bacillales*) or *Rhizomicrobium* (order *Rhizobiales*). These findings are consistent with the results of Duineveld *et al.* (2001). The authors investigated the bacterial community in the rhizosphere of chrysanthemum and found that most species were closely related to those of previously described soil bacteria such as *Pseudomonas*, *Acetobacter*, *Bacillus*, and *Arthrobacter*.

The bacterial genera *Rhizobium* and *Bradyrhizobium* are the most important dinitrogen fixers; they form symbiotic associations with specific legumes and some nonlegumes (Beauchamp *et al.*, 1997). Furthermore, there are huge numbers of free-living nitrogen-fixing diazotrophs such as *Bacillus*. Nitrogen-fixing bacteria can promote plant growth and can reduce susceptibility to diseases caused by plant pathogenic bacteria, fungi, viruses and nematodes (Kloepper *et al.*, 2004). Therefore, they are known as Plant Growth-Promoting Rhizobacteria (PGPR) (Kloepper *et al.*, 1999).

Influence of sward composition on the bacterial community structure in the rhizosphere

In the present study, the bacterial richness (number of OTUs) was negatively affected by herbicide application against dicots and monocots. In species-rich plots, higher numbers of OTUs were detected (Tab. 1). This is consistent with a study from El Fantroussi *et al.* (1999). The authors showed that different phenylurea herbicides significantly decreased the number of culturable heterotrophic bacteria in soil. In addition, Benizri and Amiaud (2005) found that the diversity of soil bacteria in fertilized grasslands increased significantly with

increasing plant diversity. The application of herbicides against dicots and/or monocots had a significant impact on many phylotypes and on *Gammaproteobacteria* and *Firmicutes* (Tab. 3, Supplemental Tab. S5). Many bacterial phylotypes were influenced by herbicide treatment against monocots and/or dicots (Tab. 1). Whereas some bacteria were significantly affected by herbicide application against dicots, other bacteria were influenced by herbicide application against monocots.

However, the sole effect of sward composition was weaker compared with the effect of sward composition in combination with mowing frequency and/or fertilization (Figs. 12A, 14C). These observations support the results of previous studies which showed that the selective effect of a certain plant species on the bacterial community in the soil or in the rhizosphere of grasslands varies with soil fertility or soil type (Bardgett *et al.*, 1999, Innes *et al.*, 2004, Harrison & Bardgett, 2010). According to Marschner *et al.* (2004), the bacterial community structure in the rhizosphere was influenced by a complex interaction between plant factors such as genotype and by different soil factors including the soil type.

The herbicide application against both dicots and monocots resulted in significant changes in plant species richness and in functional group abundances in the GrassMan experimental field (Petersen *et al.*, 2012). Plant species have been previously reported to affect specific bacterial groups in the rhizosphere (Grayston *et al.*, 1998, Costa *et al.*, 2006, Singh *et al.*, 2007, Garbeva *et al.*, 2008). Kowalchuk *et al.* (2002) found a clear plant-induced influence on bacterial community structure in the rhizosphere of non-agricultural plant species. The authors assumed that the rhizosphere selects for specific soil-borne microbial populations, resulting in a lower diversity of rhizosphere bacterial communities. In contrast to the previously reported studies, Singh *et al.* (2007) showed that the rhizosphere bacterial community composition from different plant species in grassland soils was mainly determined by soil type. The authors conclude that the influence of plant species is only weak and that there is no evidence for the selection of bacteria by plants in the rhizosphere.

Fertilization and mowing shape the bacterial community composition in the rhizosphere

We investigated the impact of different grassland management regimes on bacterial community composition in the rhizosphere by 454 pyrosequencing and by DGGE. Both methods showed that mowing frequency as well as fertilization had a strong influence on the bacterial community composition. When analyzing the effect of the parameters fertilization or mowing in combination, we found synergistic effects (Figs. 13C and 14B, Supplemental Fig. S2).

Pyrosequencing-based analyses of 16S rRNA genes revealed no significant effects of fertilization on bacterial richness in the rhizosphere (Tab. 1), but significant effects on community composition (Tab. 2, Figs. 13 and 14). These findings are in line with a study of Fierer *et al.* (2011) who observed no significant effects of N fertilization on soil bacterial diversity, but significant effects on community composition. Beauregard *et al*. (2010) found that fertilizer application led to shifts in the composition of bacterial communities without affecting their richness. In a study of soil microbial community composition and land use history in cultivated and grassland ecosystems, fertilizer and herbicide application were associated with a distinctive microbial community composition (Steenwerth *et al.*, 2002). In contrast to this, long-term fertilization regimes resulted in changes of soil bacterial community structure and diversity in northern China (Ge *et al.*, 2008).

In our study, fertilizer application had a significant impact on several bacterial phyla in the rhizosphere, for instance *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (Tab. 2). The abundance of *Acidobacteria* was significantly lower in fertilized plots. This finding corresponds to Kielak *et al.* (2008) who showed that this phylum appeared significantly lower in nutrient rich rhizosphere than in the surrounding bulk soil. In another study, the *Acidobacteria* were negative correlated with the nitrogen input level (Fierer *et al.*, 2011). This group is often considered to be oligotrophic (Fierer *et al.*, 2007, Kielak *et al.*, 2008).

Interestingly, high proportions of OTUs belonging to the *Bacteroidetes* were more abundant in fertilized plots which were mown thrice a year compared to the unfertilized plots (Fig. 13B). In addition, *Actinobacteria* were positive

correlated with the fertilization. These results are in line with a study of Fierer *et al.* (2011). The authors showed that copiotrophic taxa including members of the *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* typically increased in relative abundance in the high N plots.

Furthermore, the relative abundance of *Chloroflexi* was lower in plots with high levels of nitrogen input. This finding is in line with our study. The abundance of *Chloroflexi* decreased by fertilizer application, but only on plots mown once a year (data not shown). As mentioned before, the phylum *Nitrospirae* was significantly influenced by fertilization on the monocot-reduced plots (Tab. 2). Members of this phylum belong to the nitrite-oxidizing bacterial group. In our study, an uncultured bacterium affiliated to the *Nitrosomonadaceae* was influenced by mowing frequency and fertilization (data not shown). This is of ecological importance because the genus *Nitrosomonas* is a key player in the N cycling of soil (Acosta-Martinez *et al.*, 2008).

In addition, fertilization as well as mowing frequency had a significant impact on most phylotypes of the top 25 OTUs (Tab. 3). The combination of both treatments led to interesting results. The abundance of *Bradyrhizobium* was reduced by fertilization (Fig. 14A). However, this effect was only significant on plots mown thrice per year (Fig. 14B). In addition, the abundance was decreased by fertilization on monocot-reduced plots (Fig. 14C). As mentioned before, the bacterial genera *Bradyrhizobium* belongs to the most important dinitrogen fixers. In soils with high level of N, nodule formation is decreased (Beauchamp *et al.*, 1997) which might be explained the lower abundance of nitrogen-fixing bacteria in the rhizosphere. Furthermore, the relative abundances of soil microbial taxa associated with specific components of the soil N cycle such as nitrifiers often changes when soils are fertilized with N (Fierer *et al.*, 2011).

Effects of mowing on N fluxes and N retention in grasslands have been reported previously (Maron & Jefferies, 2001). Grazing and mowing can also affect the size and composition of key microbial functional groups driving N dynamics (Patra *et al.*, 2006). According to Denef *et al.* (2009) mowing intensity did not affect the relative abundance or activity of microbial communities in the rhizosphere of temperate grassland. This result is not consistent with the results of our study in which different mowing frequencies strongly influenced the bacteria

in the rhizosphere. The reason for these differences could be that different methodologies were used which strongly varied in phylogentic resolution.

Impact of above-ground herbivory on rhizosphere bacterial community structures

Whereas herbivory did not seem to affect the bacterial richness, although slight changes in the relative abundances of members of the *Rhizobiales*, *Frankiales*, and *Acidimicrobiales* were recorded. These findings are in line with the results of Techau *et al.* (2004) who showed that above-ground herbivory had no influence on the number of rhizosphere bacteria in pea plants.

In the present study, there was a significant interaction of the herbivory effect with fertilization and mowing (Tabs. 2 and 3). In combination with these regimes, above-ground herbivory had a significant influence on most abundant phyla such as *Actinobacteria*, *Bacteroidetes*, *Acidobacteri*a, *Proteobacteria*, *Chloroflexi*, and *Firmicutes.* In addition, the abundance of the *Actinobacteria* was significantly reduced by herbivory, but only on plots mown three times per year (Fig. 12B).

It is well-known that below-ground herbivory influences bacterial communities in the rhizosphere (Denton *et al.*, 1998, Treonis *et al.*, 2005, Poll *et al.*, 2007, Dematheis *et al.*, 2012). Denton *et al. (*1998) showed that low amounts of root herbivory (below the damage threshold) positively influence the rhizosphere microbial community in a grassland soil. According to Holland *et al.* (1995), above-ground herbivory stimulate soil bacteria at least at moderate levels of herbivory in no-tillage fields. Furthermore, grazing induces changes in the size and in the structure of bacterial communities in the soil (Northup *et al.*, 1999, Patra *et al.*, 2005). Northup *et al.* (1999) showed that grazing pressure had a stronger effect on microbial biomass than other soil or vegetative characteristics. The long-term removal of sheep grazing resulted in significant reductions in microbial biomass and activity in the surface soil while the abundance of active soil bacteria were unaffected by the removal of sheep grazing (Bardgett *et al.*, 1997).

So far, previous studies often used either cultivation-dependent approaches (Grayston *et al.*, 2001, Dawson *et al.*, 2004), microbial respiration measurements

(Holland, 1995, Bardgett *et al.*, 1997, Northup *et al.*, 1999) or cultivation-independent approaches such as DGGE (Patra *et al.*, 2005, Dematheis *et al.*, 2012) to study the effect of herbivory or grazing on the bacteria in the soil or in the rhizosphere. To our knowledge, above-ground herbivory and its influence on the bacteria in the rhizosphere have never been investigated by 454 pyrosequencing below phylum level.

Ecological significance

The effects on bacterial diversity of the studied parameters have been addressed frequently in many studies over the past years. For example, it was shown that fertilizer application influenced certain bacterial groups being involved in important nutrient cycles, e.g., the soil nitrogen cycle. Therefore, herbicide and fertilizer application as well as different mowing frequencies and above-ground herbivory are of ecological and economic importance as soil fertility is strongly affected. However, most previous studies investigated the effect of just a single biotic or abiotic factor.

The analysis conducted in this study aimed at evaluating the combined impact of different management regimes and above-ground herbivory on bacterial community structures in the rhizosphere. Although we were able to confirm the results of former studies, we also recorded discrepancies as not only a single factor but also different combinations of the studied factors influenced the abundances of several bacterial taxa in the soil.

Consequently, we have to restrict the results of former studies and their interpretation as mixed effects led to either an enhanced, reduced, or, in rare cases, opposite bacterial response. One prominent ecological example is the effect of fertilization on soil nitrogen fixation. We were able to demonstrate that fertilization does lead to a reduction of bacterial taxa capable of nitrogen fixation. However, this effect was only significant in combination with higher mowing frequencies. Therefore, fertilization does affect nitrogen fixation but only under certain circumstances.

Based on the high recorded number of mixed effects of management regimes and herbivory, versatile changes in the bacterial community composition and, correspondingly, versatile ecological outcomes can occur.

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References

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

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS, et al. (1999). Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. *Functional Ecology* 13: 650-660.

Bardgett, D. R, Leemans, K. D, Cook, et al. (1997). Seasonality of the soil biota of grazed and ungrazed hill grasslands. *Soil Biology and Biochemistry* 29: 1285-1294.

Beauchamp E, Hume D, Elsas Jv, Trevors J, Wellington E (1997). Agricultural soil manipulation: the use of bacteria, manuring, and plowing. *Modern soil microbiology*: 643-664.

Beauregard MS, Hamel C, Atul N, St-Arnaud M (2010). Long-Term Phosphorus Fertilization Impacts Soil Fungal and Bacterial Diversity but not AM Fungal Community in Alfalfa. *Microbial Ecology* 59: 379-389.

Benizri E, Amiaud B (2005). Relationship between plants and soil microbial communities in fertilized grasslands. *Soil Biology and Biochemistry* 37: 2055-2064.

Berg G, Smalla K (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology* 68: 1-13.

Bissett A, Richardson AE, Baker G, Thrall PH (2011). Long-term land use effects on soil microbial community structure and function. *Applied Soil Ecology* 51: 66-78.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7: 335-336.

Compant S, Clément C, Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms

involved and prospects for utilization. *Soil Biology and Biochemistry* 42: 669-678.

Costa R, Gotz M, Mrotzek N, Lottmann J, Berg G, et al. (2006). Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* 56: 236-249.

Cremonesi L, Firpo S, Ferrari M, Righetti PG, Gelfi C (1997). Double-gradient DGGE for optimized detection of DNA point mutations. *Biotechniques* 22: 326-330.

Dawson LA, Grayston SJ, Murray PJ, Ross JM, Reid EJ, et al. (2004). Impact of *Tipula paludosa* larvae on plant growth and the soil microbial community. *Applied Soil Ecology* 25: 51-61.

Dematheis F, Zimmerling U, Flocco C, Kurtz B, Vidal S, et al. (2012). Multitrophic interaction in the rhizosphere of maize: root feeding of Western corn rootworm larvae alters the microbial community composition. *PLoS One* 7: e37288-e37288.

Denef K, Roobroeck D, Manimel Wadu MC, Lootens P, Boeckx P (2009). Microbial community composition and rhizodeposit-carbon assimilation in differently managed temperate grassland soils. *Soil Biology and Biochemistry* 41: 144-153.

Denton CS, Bardgett RD, Cook R, Hobbs PJ (1998). Low amounts of root herbivory positively influence the rhizosphere microbial community in a temperate grassland soil. *Soil Biology and Biochemistry* 31: 155-165.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* 72: 5069-5072.

Dimkpa C, Weinand T, Asch F (2009). Plant-rhizobacteria interactions alleviate abiotic stress conditions. *Plant, Cell and Environment* 32: 1682-1694.

Doi T, Abe J, Shiotsu F, Morita S (2011). Study on rhizosphere bacterial community in lowland rice grown with organic fertilizers by using PCR-denaturing gradient gel electrophoresis. *Plant Root* 5: 5-16.

Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD, van Veen JA (2001). Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology* 67: 172-178.

Edgar RC (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26: 2460-2461.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27: 2194-2200.

El Fantroussi S, Verschuere L, Verstraete W, Top EM (1999). Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology* 65: 982-988.

Fierer N, Bradford MA, Jackson RB (2007). Toward an ecological classification of soil bacteria. *Ecology* 88: 1354-1364.

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA, et al. (2011). Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *The ISME Journal* 6: 1007-1017.

Garbeva P, Elsas JD, Veen JA (2008). Rhizosphere microbial community and its response to plant species and soil history. *Plant and Soil* 302: 19-32.

Gardner T, Acosta-Martinez V, Senwo Z, Dowd SE (2011). Soil rhizosphere microbial communities and enzyme activities under organic farming in Alabama. *Diversity* 3: 308-328.

Ge Y, Zhang J-b, Zhang L-m, Yang M, He J-z (2008). Long-term fertilization regimes affect bacterial community structure and diversity of an agricultural soil in northern China. *Journal of Soils and Sediments* 8: 43-50.

Gottel NR, Castro HF, Kerley M, Yang Z, Pelletier DA, et al. (2011). Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* 77: 5934-5944.

Grayston SJ, Dawson LA, Treonis AM, Murray PJ, Ross J, et al. (2001). Impact of root herbivory by insect larvae on soil microbial communities. *European Journal of Soil Biology* 37: 277-280.

Grayston SJ, Wang S, Campbell CD, Edwards AC (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* 30: 369-378.

Haichar FeZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, et al. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *The ISME Journal* 2: 1221-1230.

Harrison KA, Bardgett RD (2010). Influence of plant species and soil conditions on plant–soil feedback in mixed grassland communities. *Journal of Ecology* 98: 384-395.

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* 63: 3233-3241.

Holland J (1995). Effects of above-ground herbivory on soil microbial biomass in conventional and no-tillage agroecosystems. *Applied Soil Ecology* 2: 275-279.

Innes L, Hobbs P, Bardgett R (2004). The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. *Biology and Fertility of Soils* 40: 7-13.

Keuter A, Hoeft I, Veldkamp E, Corre M (2013). Nitrogen response efficiency of a managed and phytodiverse temperate grassland. *Plant and Soil* 364: 193-206.

Kielak A, Pijl AS, van Veen JA, Kowalchuk GA (2008). Phylogenetic diversity of Acidobacteria in a former agricultural soil. *The ISME Journal* 3: 378-382.

Kloepper J, Rodríguez-Kábana R, McInroy J, Young R (1992). Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: Identification by fatty acid analysis and frequency of biological control activity. *Plant and Soil* 139: 75-84.

Kloepper JW, Rodríguez-Kábana R, Zehnder AW, Murphy JF, Sikora E, et al. (1999). Plant root-bacterial interactions in biological control of soilborne

diseases and potential extension to systemic and foliar diseases. *Australasian Plant Pathology* 28: 21-26.

Kloepper JW, Ryu C-M, Zhang S (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94: 1259-1266.

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PG, van Veen JA (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek* 81: 509-520.

Lugtenberg B, Kamilova F (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* 63: 541-556.

Maron JL, Jefferies RL (2001). Restoring enriched grasslands: effects of mowing on species richness, productivity, and nitrogen retention. *Ecological Applications* 11: 1088-1100.

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

Muyzer G, de Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* 164: 165-172.

Nacke H, Thürmer A, Wollherr A, Will C, Hodac L, et al. (2011). Pyrosequencing-Based Assessment of Bacterial Community Structure Along Different Management Types in German Forest and Grassland Soils. *PLoS ONE* 6: e17000.

Northup BK, Brown JR, Holt JA (1999). Grazing impacts on the spatial distribution of soil microbial biomass around tussock grasses in a tropical grassland. *Applied Soil Ecology* 13: 259-270.

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, et al. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* 178: 5636-5643.

Nunan N, Daniell TJ, Singh BK, Papert A, McNicol JW, et al. (2005). Links between plant and rhizoplane bacterial communities in grassland soils. *Applied and Environmental Microbiology* 71: 6784-6792.

Pastorelli R, Landi S, Trabelsi D, Piccolo R, Mengoni A, et al. (2011). Effects of soil management on structure and activity of denitrifying bacterial communities. *Applied Soil Ecology* 49: 46-58.

Patra AK, Abbadie L, Clays-Josserand A, Degrange V, Grayston SJ, et al. (2006). Effects of management regime and plant species on the enzyme activity and genetic. *Environmental Microbiology* 8: 1005-1016.

Patra AK, Abbadie L, Clays-Josserand A, Degrange V, Grayston SJ, et al. (2005). Effects of Grazing on Microbial Functional Groups Involved in Soil N Dynamics. *Ecological Monographs* 75: 65-80.

Petersen U, Wrage N, Köhler L, Leuschner C, Isselstein J (2012). Manipulating the species composition of permanent grasslands-A new approach to biodiversity experiments. *Basic and Applied Ecology* 13: 1-9.

Poll J, Marhan S, Haase S, Hallmann J, Kandeler E & Ruess L (2007). Low amounts of herbivory by root-knot nematodes affect microbial community dynamics and carbon allocation in the rhizosphere. *FEMS Microbiology Ecology* 62: 268-279.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* 35: 7188-7196.

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R (2001). Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. *Crop Protection* 20: 1-11.

R Development Core Team (2012). R: A Language and Environment for Statistical Computing. Vienna, Austria: *R Foundation for Statistical Computing*.

Singh BK, Munro S, Potts JM, Millard P (2007). Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Applied Soil Ecology* 36: 147-155.

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

Sørensen J (1997). The rhizosphere as a habitat for soil microorganisms. *Modern soil microbiology*: 21-45.

Steenwerth KL, Jackson LE, Calderón FJ, Stromberg MR, Scow KM (2002). Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. *Soil Biology and Biochemistry* 34: 1599-1611.

Techau M, Bjørnlund L, Christensen S (2004). Simulated herbivory effects on rhizosphere organisms in pea (*Pisum sativum*) depended on phosphate. *Plant and Soil* 264: 185-194.

Treonis AM, Grayston SJ, Murray PJ, Dawson LA (2005). Effects of root feeding, cranefly larvae on soil microorganisms and the composition of rhizosphere solutions collected from grassland plants. *Applied Soil Ecology* 28: 203-215.

van der Heijden MG, Bardgett RD, van Straalen NM (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity. *Ecology Letters* 11: 296-310.

Wemheuer B, Güllert S, Billerbeck S, Giebel H, Voget S, et al. (2014). Impact of a phytoplankton bloom on the diversity of the active bacterial community in the German Bight as revealed by metatranscriptomic approaches. *FEMS Microbiology Ecology* 87: 378-389.

Yang CH, Crowley DE (2000). Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied and Environmental Microbiology* 66: 345-351.

Yang J, Kloepper JW, Ryu CM (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science* 14: 1-4.

Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, et al. (2002). *Mucosa*-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and Environmental Microbiology* 68: 3401-3407.

SUPPORTING INFORMATION

Tab. S1: pH values of grassland soils subjected to different fertilization and mowing regimes as well as above-ground herbivory at soil depths of 1-5 cm.

Treatment	Sward composition	Mowing	Fertilization	Herbivory	pH_{KCl}	SE	n
1	species-rich	once	no	control	4.60	0.36	6
	species-rich	once	no	herbivory	4.63	0.19	6
2	species-rich	once	NPK	control	4.87	0.29	6
	species-rich	once	NPK	herbivory	4.63	0.28	6
3	species-rich	thrice	no	control	4.63	0.17	5
	species-rich	thrice	no	herbivory	4.57	0.10	6
4	species-rich	thrice	NPK	control	4.77	0.18	6
	species-rich	thrice	NPK	herbivory	4.75	0.27	6
5	dicot-reduced	once	no	control	4.54	0.25	6
	dicot-reduced	once	no	herbivory	4.65	0.40	5
6	dicot-reduced	once	NPK	control	4.59	0.20	6
	dicot-reduced	once	NPK	herbivory	4.58	0.10	6
7	dicot-reduced	thrice	no	control	4.80	0.41	5
	dicot-reduced	thrice	no	herbivory	4.62	0.22	6
8	dicot-reduced	thrice	NPK	control	4.56	0.23	6
	dicot-reduced	thrice	NPK	herbivory	4.47	0.21	6
9	monocot-reduced	once	no	control	4.50	0.20	4
	monocot-reduced	once	no	herbivory	4.59	0.19	5
10	monocot-reduced	once	NPK	control	4.60	0.16	6
	monocot-reduced	once	NPK	herbivory	4.63	0.11	6
11	monocot-reduced	thrice	no	control	4.42	0.19	6
	monocot-reduced	thrice	no	herbivory	4.50	0.21	6
12	monocot-reduced	thrice	NPK	control	4.63	0.20	6
	monocot-reduced	thrice	NPK	herbivory	4.76	0.28	6

Tab. S2: Observed Operational Taxonomic Units (OTUs) and alpha diversity indices at 1%, 3%, and 20% genetic distances. Number of observed clusters, ACE indices, Shannon indices, Chao1 indices, and the maximal OTU number (michaelis_menten_fit index) were calculated with QIIME [63]. Coverage was determined based on observed clusters and the maximal OTU number. To compare community structures, 2,280 randomly selected sequences from each sample were used for the calculations.

Sample	*Observed OTUs*			*Max. OTU number*			*Coverage (%)*			*ACE*			*Chao1*			*Shannon*		
	80%	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*
Lys02L	124.40	613.20	708.10	168.58	1001.99	1208.45	73.79	61.20	58.60	200.63	1364.01	1525.41	195.51	1604.65	1880.80	2.99	5.63	5.86
Lys02R	137.10	707.90	812.10	184.55	1419.44	1683.01	74.29	49.87	48.25	221.31	2230.31	2553.97	220.51	2531.96	3518.24	3.09	5.63	5.88
Lys04L	117.00	588.40	615.20	152.30	878.42	920.01	76.82	66.98	66.87	217.03	973.71	986.25	201.04	1108.66	1137.23	3.17	5.71	5.79
Lys04R	102.50	470.70	516.40	128.17	662.04	741.54	79.97	71.10	69.64	162.52	784.23	897.99	154.21	938.67	1196.40	2.96	5.34	5.48
Lys06L	137.20	714.50	773.20	182.57	1301.03	1422.44	75.15	54.92	54.36	222.91	1720.44	1809.08	218.79	1933.13	2128.75	3.13	5.75	5.89
Lys06R	152.80	888.40	1010.40	195.54	2196.83	2721.60	78.14	40.44	37.13	236.11	3946.04	5273.56	254.00	4064.21	6879.47	3.42	5.96	6.20
Lys07L	131.90	751.30	863.60	175.67	1307.26	1576.65	75.08	57.47	54.77	225.19	1539.07	1722.25	210.18	1639.08	1836.72	3.18	5.96	6.20
Lys07R	127.00	689.40	784.30	166.51	1267.56	1502.44	76.27	54.39	52.20	195.70	1736.50	2026.33	188.50	2018.92	2571.20	3.02	5.62	5.88
Lys08L	169.40	967.90	1081.50	224.07	2510.03	3120.77	75.60	38.56	34.65	272.53	4289.96	5557.27	264.58	4414.91	6756.57	3.45	6.10	6.27
Lys08R	135.90	675.20	760.70	178.50	1228.68	1381.90	76.13	54.95	55.05	215.70	1690.94	1815.09	213.74	1890.07	2214.62	3.26	5.68	5.93
Lys09L	137.50	679.30	754.50	182.86	1220.89	1389.77	75.20	55.64	54.29	216.25	1798.06	1954.09	211.41	2143.97	2344.01	3.20	5.71	5.90
Lys09R	133.60	684.90	761.30	181.44	1290.96	1413.66	73.64	53.05	53.85	209.39	1790.39	1878.50	205.21	2116.47	2353.94	3.04	5.61	5.88
Lys10L	149.20	755.60	834.00	194.84	1511.56	1729.33	76.58	49.99	48.23	222.03	2450.08	2697.58	221.45	2866.43	3511.94	3.40	5.82	5.98
Lys10R	139.20	704.60	775.30	184.14	1376.68	1531.70	75.59	51.18	50.62	227.23	2376.65	2622.32	242.36	2849.38	3870.21	3.21	5.69	5.88
Lys11L	124.00	635.40	701.60	169.72	1148.32	1256.79	73.06	55.33	55.82	205.66	1741.26	1753.94	195.38	1966.87	2121.43	2.88	5.50	5.73
Lys11R	133.20	713.80	812.30	173.16	1539.47	1844.10	76.92	46.37	44.05	217.33	2854.44	3470.23	221.85	3396.18	5561.57	3.16	5.58	5.82
Lys12L	150.50	818.10	924.30	194.55	1813.60	2286.28	77.36	45.11	40.43	234.17	3111.08	4177.66	230.48	3192.01	5302.55	3.40	5.86	6.04
Lys12R	122.90	610.10	675.80	163.87	1078.12	1198.98	75.00	56.59	56.36	213.29	1612.31	1659.61	213.66	1942.62	2113.82	2.97	5.46	5.65
Lys14L	138.60	705.00	779.30	181.58	1257.44	1417.55	76.33	56.07	54.98	206.06	1781.28	2037.97	211.69	2178.10	2736.29	3.24	5.82	6.02
Lys14R	134.60	786.50	898.60	171.70	1742.72	2191.38	78.39	45.13	41.01	206.91	3195.23	4555.72	214.06	3460.09	6024.78	3.31	5.82	6.03
Lys20L	134.90	675.60	794.20	177.55	1293.59	1685.77	75.98	52.23	47.11	207.92	2052.92	2879.40	204.58	2162.22	3823.17	3.13	5.61	5.82
Lys20R	152.40	815.40	917.40	203.73	1835.56	2195.07	74.81	44.42	41.79	253.06	3370.62	3999.23	251.56	3706.62	5322.22	3.24	5.84	6.04
Lys22L	158.50	1044.80	1186.30	205.73	3436.94	4706.66	77.04	30.40	25.20	240.38	5872.04	10226.30	240.26	4992.06	8827.88	3.48	6.09	6.34
Lys22R	163.00	866.00	947.30	216.33	2030.21	2453.99	75.35	42.66	38.60	264.53	3771.14	4993.91	275.45	3903.51	6051.90	3.51	5.97	6.09
Lys23L	110.50	490.90	506.20	133.87	676.31	705.56	82.54	72.59	71.74	176.83	746.41	800.34	171.59	906.67	998.79	3.24	5.52	5.55
Lys23R	92.80	531.50	546.50	105.90	741.98	767.93	87.63	71.63	71.17	128.47	780.93	800.94	128.36	908.19	925.09	3.22	5.67	5.70
Lys27L	132.40	719.20	815.90	174.47	1412.66	1642.76	75.89	50.91	49.67	216.75	2277.72	2642.58	221.07	2790.22	3763.79	3.18	5.73	5.97
Lys27R	135.30	709.90	817.00	174.05	1375.95	1632.01	77.74	51.59	50.06	196.09	2245.04	2494.40	188.35	2766.23	3490.92	3.29	5.73	6.00
Lys28L	153.00	887.60	1017.10	199.62	2169.49	2754.93	76.65	40.91	36.92	231.84	3788.49	5404.44	223.67	4048.60	7715.37	3.40	5.95	6.23
Lys28R	131.00	755.60	858.00	168.16	1344.81	1544.56	77.90	56.19	55.55	205.04	1657.87	1752.96	196.17	1795.84	2009.55	3.28	5.96	6.25
Lys29L	126.20	629.20	720.00	169.70	1130.64	1303.95	74.37	55.65	55.22	210.40	1698.94	1735.93	206.11	1923.23	2106.96	2.98	5.50	5.75
Lys29R	128.40	639.90	724.70	178.79	1120.06	1306.53	71.81	57.13	55.47	221.85	1528.13	1734.60	212.56	1718.57	2087.78	2.93	5.58	5.79
Lys30L	142.20	688.10	768.20	192.62	1249.57	1416.22	73.82	55.07	54.24	242.86	1817.90	1909.38	230.97	2103.55	2279.68	3.20	5.67	5.88

Tab. S2: continued.

Sample	*Observed OTUs*			*Max. OTU number*			*Coverage (%)*			*ACE*			*Chao1*			*Shannon*		
	80%	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*
Lys30R	159.90	822.30	957.50	211.67	1868.18	2380.07	75.54	44.02	40.23	247.76	2934.14	3649.31	247.42	3179.95	4523.74	3.48	5.80	6.07
Lys31L	133.70	662.90	755.60	175.30	1211.72	1441.56	76.27	54.71	52.42	210.89	1646.03	1861.35	218.73	1866.55	2207.82	3.22	5.53	5.74
Lys31R	132.70	648.90	764.20	171.16	1205.28	1483.60	77.53	53.84	51.51	207.86	1923.17	2111.38	203.92	2206.34	2692.24	3.15	5.50	5.79
Lys33L	101.40	457.30	553.50	133.58	748.86	933.45	75.91	61.07	59.30	152.87	1211.14	1444.46	151.82	1352.07	1852.43	2.65	4.94	5.29
Lys33R	120.10	617.90	712.40	157.86	1100.85	1288.07	76.08	56.13	55.31	181.78	1570.99	1716.97	176.89	1733.68	2053.00	2.94	5.47	5.76
Lys36L	121.40	587.60	655.60	168.20	1025.58	1128.25	72.18	57.29	58.11	206.25	1563.93	1589.38	200.20	1808.04	1996.19	2.88	5.41	5.63
Lys36R	135.80	699.90	786.60	183.16	1388.37	1535.81	74.14	50.41	51.22	217.95	2130.46	2269.74	217.68	2526.99	3225.60	2.99	5.59	5.86
Lys37L	135.00	756.80	839.30	175.67	1478.60	1670.54	76.85	51.18	50.24	198.00	2370.97	2616.99	197.81	3006.85	3754.14	3.15	5.84	6.08
Lys37R	126.10	724.30	854.10	163.10	1733.00	2165.42	77.32	41.79	39.44	200.23	3385.80	4839.11	199.01	3146.18	6147.46	3.04	5.49	5.86
Lys38L	126.90	663.00	743.00	165.25	1125.51	1286.48	76.79	58.91	57.75	213.20	1501.90	1584.54	216.94	1655.79	1789.88	3.20	5.75	5.96
Lys38R	139.00	721.70	802.00	184.79	1309.40	1474.78	75.22	55.12	54.38	211.09	1863.92	1938.11	201.15	2127.29	2312.06	3.25	5.84	6.07
Lys40L	125.10	712.00	833.60	156.18	1502.08	1823.71	80.10	47.40	45.71	185.73	2676.19	3399.58	188.27	2700.32	5013.58	3.18	5.63	5.95
Lys40R	127.60	723.30	831.80	164.26	1488.83	1778.35	77.68	48.58	46.77	199.59	2489.79	2989.29	201.00	2974.71	4460.57	3.15	5.68	5.94
Lys41L	125.40	615.90	703.80	159.45	1002.48	1185.80	78.65	61.44	59.35	205.52	1246.04	1402.96	201.36	1356.61	1530.74	3.24	5.63	5.86
Lys41R	156.60	813.80	925.70	202.90	1807.92	2166.17	77.18	45.01	42.73	240.51	3089.32	3659.52	236.19	3607.25	4952.03	3.51	5.85	6.10
Lys43L	115.70	628.70	717.80	150.10	1089.98	1234.26	77.08	57.68	58.16	193.00	1430.94	1492.82	191.49	1540.46	1695.69	3.02	5.56	5.86
Lys43R	118.00	658.20	793.30	153.20	1193.55	1524.10	77.02	55.15	52.05	186.36	1617.69	1832.38	182.10	1712.27	2019.81	3.05	5.59	5.91
Lys44L	140.90	709.00	798.90	190.02	1395.81	1609.61	74.15	50.79	49.63	240.00	2218.70	2405.03	233.06	2723.59	3292.70	3.22	5.67	5.88
Lys44R	136.80	784.90	921.00	170.16	1902.55	2645.66	80.39	41.26	34.81	204.75	3272.74	5810.00	199.75	3008.28	5959.91	3.42	5.62	5.87
Lys47L	134.40	690.50	792.50	174.07	1408.84	1679.25	77.21	49.01	47.19	198.52	2296.38	2663.35	197.20	2696.86	3651.99	3.14	5.54	5.80
Lys47R	122.20	655.40	793.70	154.22	1064.94	1361.81	79.24	61.54	58.28	204.11	1206.90	1420.85	202.18	1274.13	1493.25	3.27	5.77	6.13
Lys48L	149.90	921.60	1085.00	187.62	2496.16	3473.12	79.90	36.92	31.24	219.16	4450.74	7679.82	211.54	4037.66	8800.12	3.48	5.94	6.24
Lys48R	147.00	750.50	844.20	191.83	1523.18	1793.60	76.63	49.27	47.07	222.68	2558.71	3014.31	215.48	2977.90	4259.41	3.39	5.77	5.97
Lys55L	148.70	814.50	942.10	192.05	1761.81	2218.63	77.43	46.23	42.46	238.54	2975.24	3895.65	224.93	3372.36	5482.30	3.37	5.88	6.14
Lys55R	140.40	741.30	838.10	186.10	1760.34	2084.64	75.44	42.11	40.20	206.06	3492.35	4646.39	202.29	3289.92	6285.88	3.13	5.58	5.82
Lys56L	135.70	637.30	750.90	177.98	1052.85	1271.97	76.25	60.53	59.03	211.47	1328.26	1455.68	202.84	1438.20	1641.13	3.20	5.68	6.02
Lys56R	110.10	562.30	697.70	137.60	859.05	1119.90	80.02	65.46	62.30	174.74	1004.28	1197.72	173.05	1091.27	1320.93	3.10	5.51	5.93
Lys62L	141.90	782.70	876.00	184.88	1826.56	2066.45	76.75	42.85	42.39	227.65	3248.01	3849.42	237.84	3886.90	5951.37	3.26	5.69	5.96
Lys62R	140.90	774.70	871.60	185.74	1658.70	1912.94	75.86	46.71	45.56	215.88	2873.92	3211.71	208.47	3440.93	4916.07	3.16	5.77	6.02
Lys64L	125.70	667.20	787.60	167.86	1145.97	1426.57	74.88	58.22	55.21	213.54	1368.42	1582.89	205.35	1340.94	1571.61	3.04	5.73	6.00
Lys64R	126.10	672.00	778.60	166.15	1179.30	1395.59	75.90	56.98	55.79	202.40	1486.07	1614.79	191.90	1602.96	1731.96	3.12	5.69	5.98
Lys67L	149.60	873.40	992.00	189.61	2244.28	2736.72	78.90	38.92	36.25	212.07	4207.05	5790.60	205.10	3836.27	8024.21	3.39	5.87	6.12
Lys67R	150.40	808.50	927.80	192.67	1722.22	2139.62	78.06	46.95	43.36	216.99	2812.65	3704.16	211.75	3374.15	5217.90	3.47	5.89	6.13
Lys68L	152.70	961.00	1096.60	200.33	2885.49	3977.67	76.22	33.30	27.57	230.58	5538.82	9245.21	234.93	5112.44	10786.18	3.41	5.95	6.16
Lys68R	136.80	714.80	801.60	179.15	1406.60	1545.16	76.36	50.82	51.88	210.06	2322.89	2309.22	200.34	3011.73	3235.55	3.20	5.71	6.00
Lys70L	140.00	706.50	776.60	185.02	1346.22	1496.11	75.67	52.48	51.91	226.09	2112.45	2286.81	215.77	2612.04	3119.94	3.22	5.72	5.93
Lys70R	146.60	814.10	924.50	193.73	1889.15	2183.08	75.67	43.09	42.35	225.60	3336.21	3884.92	222.32	3623.32	5935.47	3.22	5.82	6.10

Tab. S2: continued.

Sample	*Observed OTUs*			*Max. OTU number*			*Coverage (%)*			*ACE*			*Chao1*			*Shannon*		
	80%	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*	*80%*	*97%*	*99%*
Lys71L	147.80	761.40	821.50	197.19	1502.13	1657.54	74.95	50.69	49.56	221.85	2440.77	2625.47	223.65	3046.96	3523.22	3.18	5.84	5.97
Lys71R	152.30	842.00	948.00	198.32	2204.90	2629.08	76.80	38.19	36.06	229.33	4194.41	5540.61	238.22	4332.39	8038.56	3.29	5.70	5.99

Tab. S3: Relative abundances of abundant bacterial phyla and proteobacterial classes with respect to the different treatments (Supplemental Tab. S1) and the above-ground herbivory (c=control, h=herbivory).

Treatment	**1**		**3**		**2**		**4**		**6**		**7**		**5**		**8**		**9**		**11**		**10**		**12**		**Mean value (%)**
Phyla	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	
Acidobacteria	0.26	0.24	0.27	0.3	0.24	0.21	0.22	0.24	0.27	0.26	0.25	0.22	0.22	0.22	0.22	0.35	0.23	0.22	0.22	0.24	0.21	0.25	0.24	0.3	24.63
Actinobacteria	0.15	0.15	0.17	0.13	0.16	0.18	0.19	0.16	0.13	0.13	0.15	0.15	0.2	0.18	0.17	0.13	0.16	0.15	0.21	0.19	0.17	0.15	0.17	0.15	16.16
Bacteroidetes	0.06	0.08	0.05	0.05	0.05	0.07	0.06	0.07	0.07	0.08	0.07	0.07	0.05	0.05	0.07	0.04	0.06	0.08	0.05	0.04	0.07	0.06	0.05	0.07	6.18
Chloroflexi	0.03	0.03	0.03	0.02	0.02	0.03	0.04	0.03	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	2.97
Firmicutes	0.04	0.03	0.03	0.03	0.02	0.02	0.03	0.02	0.05	0.03	0.03	0.06	0.06	0.04	0.06	0.04	0.06	0.03	0.05	0.04	0.04	0.02	0.03	0.02	3.59
Gemmatimonadetes	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.04	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.02	0.04	0.04	0.03	0.03	2.98
Alphaproteobacteria	0.21	0.22	0.21	0.21	0.25	0.23	0.21	0.22	0.19	0.22	0.2	0.2	0.22	0.26	0.22	0.22	0.21	0.21	0.25	0.24	0.24	0.21	0.21	0.19	21.77
Betaproteobacteria	0.08	0.08	0.08	0.08	0.07	0.08	0.09	0.08	0.07	0.07	0.08	0.08	0.05	0.05	0.04	0.05	0.08	0.1	0.06	0.07	0.06	0.08	0.08	0.08	7.27
Gammaproteobacteria	0.03	0.04	0.04	0.04	0.06	0.05	0.04	0.05	0.05	0.04	0.04	0.03	0.04	0.05	0.06	0.04	0.04	0.05	0.04	0.03	0.06	0.06	0.06	0.05	4.72
Deltaproteobacteria	0.06	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.05	0.05	0.05	0.05	0.06	0.07	0.05	0.05	0.05	0.06	0.06	0.05	5.59
other bacteria	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.06	0.06	0.03	0.04	0.05	0.04	0.04	0.04	0.04	0.03	0.03	0.04	0.04	0.03	4.13

Tab. S4: Relative abundances of rare bacterial phyla with respect to the different treatments (Supplemental Tab. S1) and the above-ground herbivory (c=control, h=herbivory).

Treatment	**1**		**3**		**2**		**4**		**6**		**7**		**5**		**8**		**9**		**11**		**10**		**12**		**Mean value (%)**
Phyla	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	c	h	
Armatimonadetes	1E-04	3E-04	5E-05	0	6E-05	3E-04	3E-04	3E-04	1E-04	5E-05	3E-04	4E-04	9.2E-05	2E-04	3E-04	2E-04	8E-05	2E-04	9E-05	1E-04	0	4E-04	2E-04	1E-04	0.018
BD1-5	6E-05	4E-04	0	6E-05	6E-05	8E-05	2E-04	6E-05	1E-04	0	1E-04	0	7E-05	5E-05	1E-04	0	4E-05	3E-05	4E-05	1E-04	0	0	2E-04	0	0.008
BHI80-139	2E-04	3E-04	2E-04	4E-04	0	6E-05	2E-04	7E-05	3E-05	1E-04	1E-04	5E-04	0	7E-05	0	9E-05	2E-04	2E-04	2E-04	0	0	0	0	4E-05	0.012
Candidate division BRC1	1E-04	2E-04	0	6E-05	1E-04	1E-04	3E-04	0	5E-05	0	4E-05	1E-04	0.00014	4E-05	7E-05	0	4E-04	0	1E-04	0	7E-05	7E-05	0	1E-04	0.009
Candidate division OD1	5E-04	3E-04	1E-04	3E-04	7E-04	5E-04	1E-04	3E-04	6E-04	5E-04	5E-04	3E-04	0.00047	4E-04	6E-04	7E-04	3E-04	2E-04	2E-04	1E-04	1E-04	4E-04	4E-04	4E-05	0.036
Candidate division OP11	5E-04	7E-05	1E-04	0	2E-04	2E-04	5E-04	4E-04	8E-04	5E-04	6E-04	4E-04	0.0003	6E-04	0.002	7E-04	4E-05	2E-04	2E-04	0	3E-04	1E-04	2E-04	7E-05	0.038
Candidate division OP3	2E-04	2E-04	5E-05	1E-04	5E-05	1E-04	0	0	1E-04	1E-04	2E-04	3E-04	6.5E-05	9E-05	2E-04	2E-04	8E-05	1E-04	9E-05	6E-05	0	0	1E-04	1E-04	0.011
Candidate division TM7	4E-04	4E-04	1E-04	6E-05	6E-04	3E-04	0.001	5E-04	3E-04	7E-04	4E-04	4E-04	0.00026	6E-04	7E-04	4E-04	5E-04	7E-04	3E-04	9E-05	3E-04	7E-04	5E-04	2E-04	0.044
Candidate division WS3	0.003	0.005	0.01	0.007	0.001	0.003	0.005	0.005	0.004	0.007	0.005	0.01	0.0048	0.002	0.001	0.001	0.006	0.005	0.003	0.003	0.002	9E-04	0.002	0.002	0.408
Candidate division WS6	0	0	0	0	6E-05	0	0	0	0	3E-05	8E-05	1E-04	3.8E-05	0	2E-04	3E-04	0	3E-05	0	0	0	6E-05	0	4E-05	0.004
Chlamydiae	0	0	0	0	0	0	5E-05	0	0	0	1E-04	0	0	4E-05	2E-04	7E-05	8E-05	0	2E-04	0	0	0	0	0	0.003
Chlorobi	0.002	0.002	0.001	9E-04	0.002	0.002	0.001	0.001	0.003	0.002	0.002	0.002	0.00167	0.002	0.002	0.003	0.002	0.001	0.002	0.001	0.002	0.002	0.001	0.002	0.185
Cyanobacteria	0.004	0.004	0.002	0.005	0.006	0.005	0.005	0.005	0.004	0.005	0.005	0.004	0.003	0.004	0.006	0.008	0.003	0.003	0.003	0.004	0.004	0.005	0.005	0.005	0.446
Deferribacteres	0	0	0	0	0	0	0	0	0	6E-05	0	0	0	0	0	0	7E-05	0	0	0	0	0	0	0	0.001
Deinococcus-Thermus	0	0	0	0	0	6E-05	0	0	0	3E-05	0	0	0	0	0	0	0	0	0	0	6E-05	0	0	0	0.001
Elusimicrobia	0.005	0.005	0.004	0.006	0.005	0.005	0.003	0.004	0.006	0.006	0.007	0.004	0.00434	0.005	0.006	0.004	0.004	0.006	0.003	0.004	0.003	0.007	0.006	0.003	0.476
Fibrobacteres	0.002	0.002	0.002	0.001	0.003	0.003	0.001	0.003	0.003	0.003	0.002	0.002	0.00189	0.002	0.002	0.001	9E-04	0.003	8E-04	0.001	0.003	0.003	0.002	0.002	0.211

Tab. S4: continued.

Treatment	1		3		2		4		6		7		5		8		9		11		10		12		Mean value (%)
Fusobacteria	0	0	5E-05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000
Kazan-3B-28	0	0	5E-05	0	1E-04	0	4E-04	0	0	0	0	0	3E-05	0	0	4E-05	0	0	0	0	0	0	0	0	0.002
MVP-21	6E-05	0	0	0	0	4E-05	0	1E-04	0	0	0	0	0	0	1E-04	0	5E-05	0	0	0	0	0	0	4E-05	0.002
NPL-UPA2	0	0	0	0	0	0	0	0	0	0	0	1E-04	0	0	0	0	0	0	0	0	0	0	0	0	0.001
Nitrospirae	0.005	0.005	0.005	0.006	0.004	0.003	0.007	0.003	0.005	0.005	0.005	0.008	0.00396	0.003	0.002	0.005	0.008	0.005	0.004	0.003	0.004	0.002	0.003	0.003	0.454
Planctomycetes	0.01	0.008	0.007	0.004	0.004	0.007	0.007	0.002	0.004	0.005	0.011	0.013	0.00469	0.004	0.01	0.006	0.006	0.005	0.01	0.006	0.002	0.006	0.004	0.003	0.622
SM2F11	8E-04	0.002	0.001	0.001	0.002	7E-04	0.001	0.002	0.003	0.003	0.003	0.002	0.00116	0.001	0.002	0.002	0.002	0.002	0.001	0.002	8E-04	0.001	0.001	9E-04	0.159
Spirochaetes	2E-04	6E-04	3E-04	3E-04	0.001	5E-04	1E-04	4E-04	9E-04	6E-04	7E-04	6E-04	0.00026	2E-04	1E-04	4E-04	2E-04	5E-04	1E-04	2E-04	5E-04	3E-04	8E-04	7E-04	0.044
TA06	0	0	0	0	0	1E-04	1E-04	7E-05	0	0	0	0	7E-05	1E-04	0	5E-05	5E-05	0	5E-05	0	1E-04	0	5E-05	1E-04	0.004
TM6	9E-04	9E-04	7E-04	4E-04	6E-04	6E-04	7E-04	8E-04	0.001	8E-04	9E-04	9E-04	0.00032	6E-04	0.001	9E-04	5E-04	9E-04	3E-04	5E-04	8E-04	3E-04	0.001	1E-03	0.075
Tenericutes	0	7E-05	0	0	0	0	0	0	0	1E-04	0	0	0	0	0	0	0	0	0	0	0	1E-04	0	0	0.001
Thermotogae	0	0	5E-05	0	1E-04	0	2E-04	3E-04	1E-04	1E-04	1E-04	0	0	0	3E-05	4E-05	1E-04	0	9E-05	0	0	1E-04	2E-04	4E-05	0.007
Verrucomicrobia	0.006	0.007	0.002	0.002	0.005	0.005	0.002	0.001	0.005	0.004	0.008	0.006	0.00422	0.005	0.007	0.004	0.003	0.004	0.004	0.002	0.003	0.004	0.004	0.002	0.420
WCHB1-60	0.003	0.003	0.001	0.002	0.002	0.002	0.002	0.002	0.001	0.001	0.002	9E-04	0.0017	0.002	0.005	0.002	0.003	0.002	0.002	0.002	0.003	0.002	0.002	0.002	0.206

Tab. S5: Relative abundances and taxonomic affiliations of the 25 most abundant OTUs with respect to the different treatments (Supplemental Tab. S1) and the above-ground herbivory (c=control, h=herbivory).

OTU ID	taxonomic affiliation	Rel. abundance (%)
15254	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae;Bradyrhizobium;Bradyrhizobium sp.	4.80
3020	Bacteria;Acidobacteria;Acidobacteria;Candidatus Solibacter;uncultured Acidobacteria bacterium	1.95
15334	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Xanthobacteraceae;uncultured;bacterium Ellin6561	1.90
430	Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;Candidatus Koribacter;uncultured bacterium	1.75
31887	Bacteria;Firmicutes;Bacilli;4-15;uncultured Bacillaceae bacterium	1.34
10498	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;alphaI cluster;uncultured bacterium	1.13
33544	Bacteria;Acidobacteria;Acidobacteria;DA052;uncultured bacterium	1.07
30880	Bacteria;Acidobacteria;Acidobacteria;Order Incertae Sedis;Family Incertae Sedis;Bryobacter;uncultured Acidobacteria bacterium	1.05
26950	Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;uncultured;uncultured bacterium	1.03
15204	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Family Incertae Sedis;Rhizomicrobium;uncultured bacterium	0.97
35896	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;uncultured;uncultured bacterium	0.91
1434	Bacteria;Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Chitinophagaceae;uncultured;uncultured bacterium	0.91
43557	Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;uncultured;uncultured bacterium	0.88
10655	Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Flexibacter;uncultured bacterium	0.84
42418	Bacteria;Actinobacteria;Actinobacteria;Frankiales;Acidothermaceae;Acidothermus;uncultured bacterium	0.79
10041	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Beijerinckiaceae;uncultured;uncultured proteobacterium	0.79
23893	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;uncultured;uncultured bacterium	0.74
24136	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Xanthobacteraceae;uncultured;uncultured bacterium	0.72
17761	Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;uncultured;uncultured bacterium	0.68
10097	Bacteria;Acidobacteria;Acidobacteria;Candidatus Solibacter;uncultured Acidobacteria bacterium	0.67
1655	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;DA111;uncultured bacterium	0.63
30883	Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;uncultured;uncultured beta proteobacterium	0.61
41226	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;DA111;uncultured bacterium	0.61
22924	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;JG37-AG-20;uncultured Rhodospirillaceae bacterium	0.60
38931	Bacteria;Acidobacteria;Acidobacteria;Candidatus Solibacter;uncultured Acidobacteria bacterium	0.58

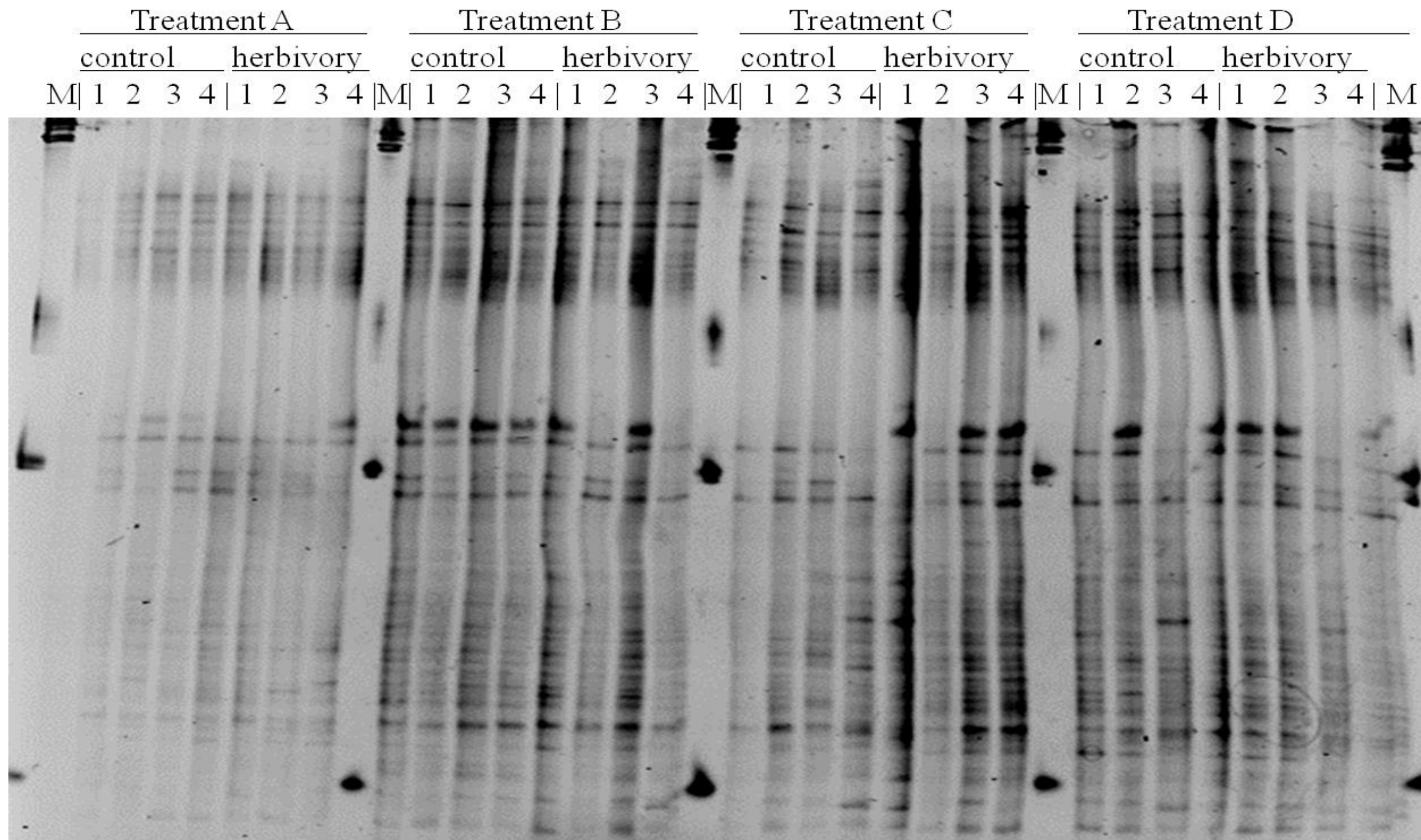

Fig.S1. 16S-DGGE profile of dicot-reduced plots showing the influence of different fertilization and mowing regimes as well as above-ground herbivory on bacterial endophyte communities in the rhizosphere. Soil samples were taken in summer 2011. Independent replicates are indicated with numbers from 1 to 4. Treatment A: 1 x mowing/ year, no NPK; treatment B: 3 x mowing/ year, no NPK; treatment C: 1 x mowing/ year, NPK; treatment D: 3 x mowing/ year, NPK. M: GeneRuler *1 kb* DNA *Ladder* (Fermentas, St. Leon-Rot, Germany).

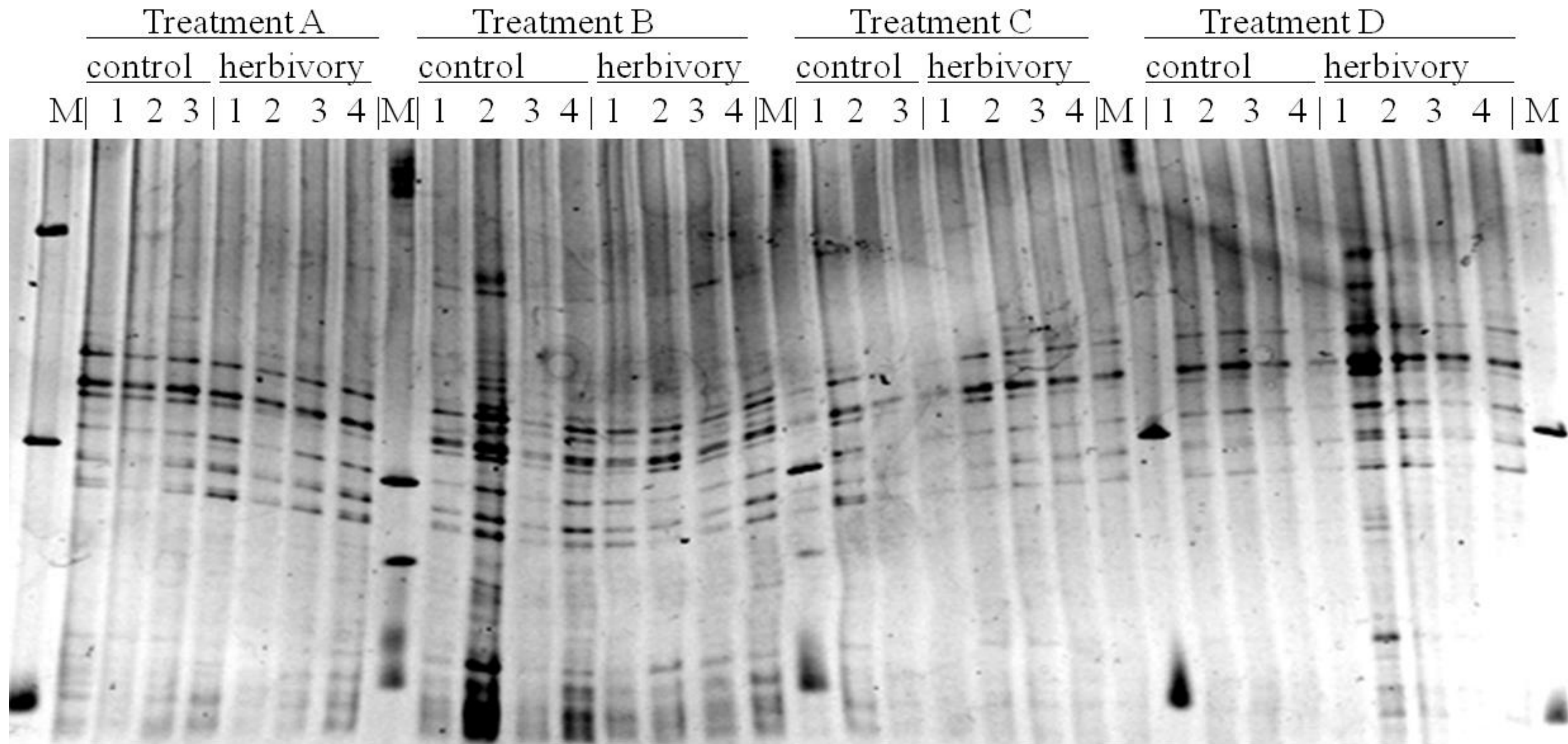

Fig.S2. 16S-DGGE profile of dicot-reduced plots showing the influence of different fertilization and mowing regimes as well as above-ground herbivory on bacterial endophyte communities in the rhizosphere. For further details see Fig. S1.

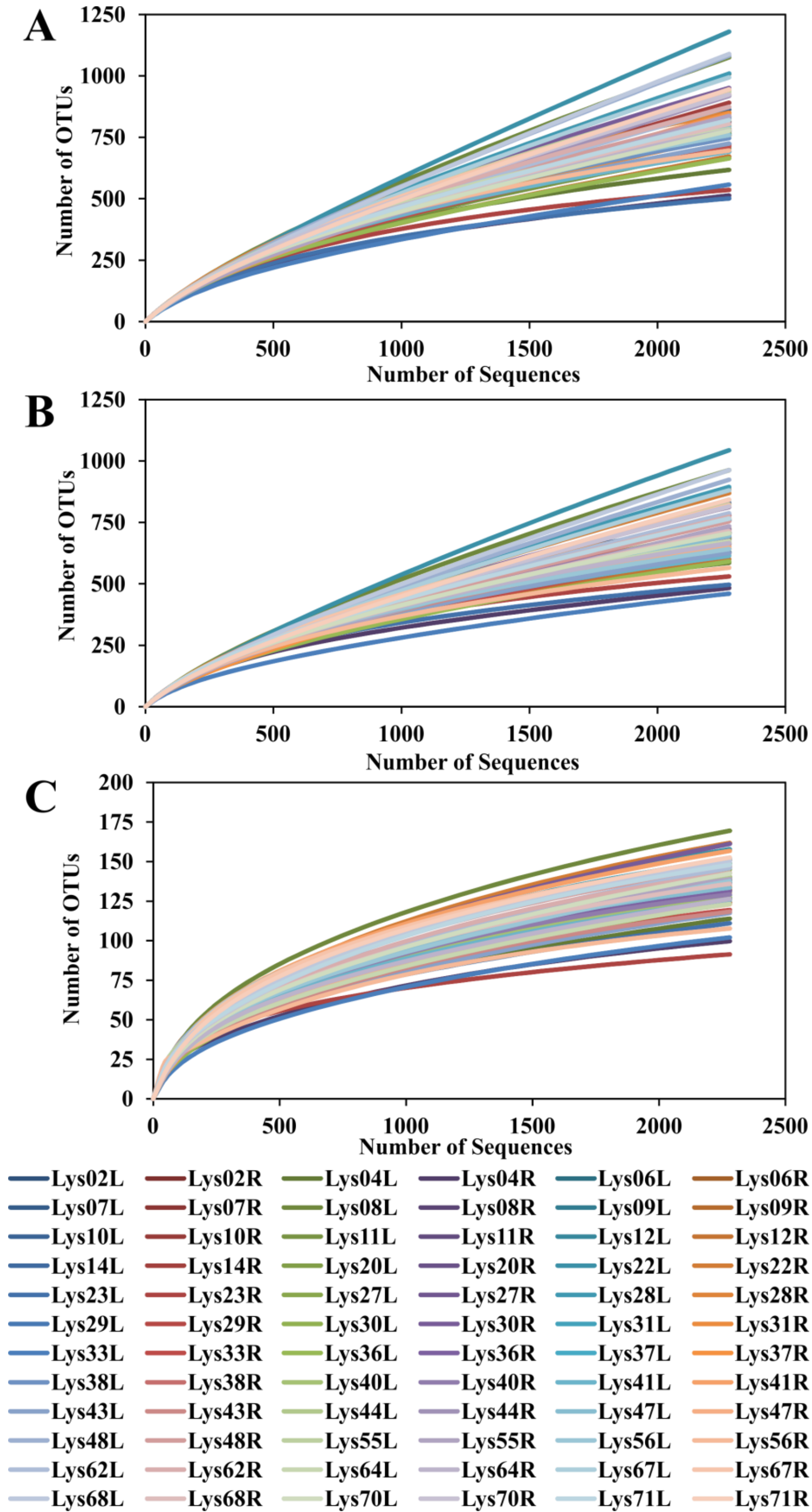

Fig. S3. Rarefaction Curves at 99% (A), 97% (B), and 99% (C) for all 72 samples analyzed by pyrosequencing. Curves were calculated in QIIME (Caporaso *et al.*, 2010).

CHAPTER D

GENERAL DISCUSSION

This study aimed at complementing the results of an ongoing research of plant-associated bacteria. Specifically, we investigated the influence of management regimes on plant-associated bacteria in the endosphere (Chapter 2) and in the rhizosphere (Chapter 3) using culture-independent molecular techniques. In addition, we analyzed the impact of above-ground herbivory on the bacterial diversity in the rhizosphere (Chapter 3). All investigations were carried out in the same area, the Grassland Management Experiment (GrassMan) in the Solling Mountains, central Germany.

So far, studies dealing with the effects of management regimes on bacterial communities in the rhizosphere or the soil have been carried out in either sown experimental fields or in greenhouse experiments. In contrast to these studies, the GrassMan project was set-up as a long-term field experiment. It was established at a semi-natural, moderately species-rich grassland site. To the best of our knowledge, this is the first study about plant-associated bacteria performed in a field experiment with a combination of different grassland management regimes.

1 PHYLOGENETIC ANALYSIS OF PLANT-ASSOCIATED BACTERIA IN THE ENDOSPHERE OF *L. PERENNE*, *F. RUBRA*, AND *D. GLOMERATA* UNDER DIFFERENT MANAGEMENT REGIMES

In Chapter 2 we showed that the bacterial endophytic community composition differed between different grassland management regimes when analyzing the effects for the specific grass species *L. perenne*, *F. rubra*, and *D. glomerata*, respectively. Interestingly, the grass species responded differently on fertilization and mowing treatments. For example, fertilizer application had a high impact on endophytic bacterial diversity in tillers of *L. perenne* and *F. rubra*. Moreover, mowing had an effect on the bacterial community composition in tillers of *L. perenne* derived from unfertilized plots.

In contrast to these findings, the community structure of *D. glomerata* was not influenced by either mowing or fertilization. This result is consistent with the findings of Seghers *et al.* (2004), who showed that the application of herbicides as well as mineral fertilizer had no impact on the bacterial endophytic community structure in the kernels of maize. However, in the same study, different types of

fertilizer (compost vs. mineral fertilizer) resulted in different root endophytic communities. Their findings correspond with the results of Fuentes-Ramírez *et al.* (1999). They found that high nitrogen-fertilization led to a reduced colonization by *Gluconacetobacter diazotrophicus* in sugarcane. The authors concluded that nitrogen supply altered the plant physiology state, thus, influencing the endophytic population growth and the interaction between plant and endophyte.
In a study using rice, a rapid change of the population and the activity of root-associated nitrogen-fixing bacteria were observed within 15 days after N-fertilization (Tan *et al.*, 2003). Moreover, the bacterial endophytic community in crops is also influenced by organic amendments to plants (Hallmann *et al.*, 1999). The authors hypothesized that changes in the physiology of the host plants may result in the development of distinct bacterial endophytic populations.

2 SEASONAL EFFECT ON BACTERIA IN THE ENDOSPHERE OF *L. PERENNE*, *F. RUBRA*, AND *D. GLOMERATA*

In this thesis, we were also able to prove a seasonal effect on the bacterial endophyte community. This result is consistent with a study of McInroy and Kloepper (1995) who showed that the bacterial endophytic population in sweet corn and cotton changes according to the season. In another study with soybean, plant age and the sampling time had an effect on the presence of bacterial endophytic species (Kuklinsky-Sobral *et al.*, 2004).

The increase of endophytic bacteria during plant development is in line with the hypothesis that endophytic bacteria colonize plants from either the rhizosphere or the soil. Vertical transmission via the seeds would either result in a constant or decreasing number of endophytic species. Furthermore, as seasonal changes in soil and rhizosphere microbial communalities have been reported previously (Smalla *et al.*, 2001, Dunfield & Germida, 2003, Habekost *et al.*, 2008, Houlden *et al.*, 2008), these changes would consequently impact the endophytic colonization of plants growing in these soils.

3 GRASSLAND MANAGEMENT REGIMES SHAPE THE BACTERIAL COMMUNITY IN THE RHIZOSPHERE

In chapter 3, we investigated the influence of different grassland management regimes on the bacterial community structure in the rhizosphere. We found distinct pattern indicating that different mowing frequencies and fertilization application as well as sward composition significantly influenced bacterial communities in the rhizosphere. The reduction of plant diversity led to a reduction in bacterial richness. A lower number of OTUs was recorded in both monocot-reduced and dicot-reduced plots. This is in accordance with a study of Benizri and Amiaud (2005) who showed that the diversity of soil bacteria increased with increasing plant diversity. In a study of El Fantroussi *et al.* (1999), herbicides significantly reduced the number of culturable heterotrophic bacteria in the soil. However, the sward composition had a weaker influence on the bacterial richness compared to the effect of sward composition in combination with mowing frequency and/or fertilizer applications. These observations support the results of Bardgett *et al.* (1999) and Innes *et al.* (2004) who showed that the effect of bacterial selection by plants can vary with soil fertility.

Higher mowing frequencies resulted in an increased bacterial richness in the rhizosphere, while fertilization did not significantly impact the bacterial richness. However, the bacterial community composition was significantly affected by the management regimes studied. Whereas sward composition as well as different mowing frequencies had no influence on the overall community composition, fertilization application had a strong impact on the community composition. Further analyses revealed that a variety of distinct bacterial groups and species specifically react to the parameters manipulated in this experiment. For example, the *Acidobacteria* were less abundant on fertilized plots. This finding is in accordance with Kielak *et al.* (2008) who showed that this phylum was detected significantly less in the nutrient rich rhizosphere than in the surrounding bulk soil. Furthermore, *Acidobacteria* were negatively correlated with the nitrogen input level (Fierer *et al.*, 2011).

Not only fertilizer applicationn but also the different mowing frequencies influenced the abundance of certain bacterial species. This effect was stronger in combination with fertilization. For example, the abundance of *Verrucomicrobia*

was significantly affected by fertilization and mowing but not by fertilization or mowing only. According to Patra *et al.* (2006), grazing and mowing can affect the size and composition of key microbial functional groups driving N dynamics. In contrast to the present study, Denef *et al.* (2009) found that mowing intensity did not affect the relative abundance or activity of microbial communities in the rhizosphere. These contrasting results might be due to the experimental setup or to the different methods used in the studies. Furthermore, the experimental sites exhibited various land use histories and soil types. It is well-known that soil type (Singh *et al.*, 2007, Garbeva *et al.*, 2008) as well as land use history (Garbeva *et al.*, 2008) influence the bacterial community structure in the rhizosphere.

The sward composition also affected certain bacterial groups, e.g., *Firmicutes* were more abundant on herbicide-treated plots than on the control plots. Plant species have been previously reported to influence specific bacterial groups in the rhizosphere (Smalla *et al.*, 2001, Costa *et al.*, 2006, Garbeva *et al.*, 2008). Kowalchuk *et al.* (2002) found a clear plant-induced influence on the bacterial community structure in the rhizosphere when comparing non-agricultural plant species. Furthermore, the authors assumed that the rhizosphere selects for specific soil-borne microbial populations.

According to Garbeva *et al.* (2008), plant species had a strong effect on the bacterial community and diversity. In contrast to these results, Singh *et al.* (2007) showed that the community structure of bacteria was mainly influenced by soil type and not by plant species. The authors conclude that the influence of plant species is only weak and that there is no evidence for plant species selection of microbial communities in the rhizosphere of different grass species.

4 EFFECT OF ABOVE-GROUND HERBIVORY ON BACTERIAL COMMUNITY COMPOSITION IN THE RHIZOSPHERE

Finally, we also investigated the effect of short-term above-ground herbivory on the bacterial community composition in the rhizosphere. Whereas herbivory did not seem to affect bacterial richness as well as the overall bacterial community composition, slight changes were recorded for some bacterial species. These findings are in line with the results of Techau *et al*. (2004) who showed that above-ground herbivory had no influence on the number of rhizosphere bacteria.
According to Bardgett *et al.* (1997), sheep grazing resulted in significant reductions in microbial biomass and activity in the surface soil while the abundance of active soil bacteria were unaffected by the removal of sheep grazing. This is consistent with our study; no direct correlation between above-ground herbivory and the abundance of predominant bacterial groups was recorded.

When analyzing the impact of herbivory alone, an uncultured *Acidobacterium* was significantly affected. However, in combination with fertilization and mowing, herbivory had a significant influence on the most abundant phyla. For example, the abundance of *Actinobacteria* was significantly reduced by above-ground herbivory but only in plots mown three times per year.
According to Holland (1995), above-ground herbivory stimulates microbial respiration and therefore soil bacteria at least at moderate levels of herbivory. Denton *et al.* (1998) showed that low amounts of root herbivory (below the damage threshold) resulted in significant increases in total microbial biomass and in the abundance of gram-positive and gram-negative specific PLFAs in the rhizosphere soil. However, both recorded increases do not indicate a higher microbial diversity. They only indicate a higher cell number.

5 CONCLUDING REMARKS

To our knowledge this is the first study investigating the influence of combined different grassland management regimes on both endophytic bacteria and bacteria in the plant rhizosphere in a field experiment.

Our results demonstrate that mowing and fertilization affect certain bacterial endophytes in the investigated grass species. However, this influence varies between different grass species analyzed. As seasonal samplings also impacted endophytic community composition, the impact of different management regimes did change with time. The effect of plant species and season should be considered in further studies as they might alter the endophytic response. As consequence, samples from different plant species collected at different time points should be analyzed when investigating the impact of different factors such as management regimes on the bacterial community composition in the endosphere.

The bacterial community composition in the rhizosphere was also influenced by different grassland management regimes. Moreover, above-ground herbivory appears to have a minor influence on the community composition. In addition, the combination of herbivory with mowing, fertilization, and sward composition had significant effects on the community composition. These interactions enhanced, reduced, or neutralized the recorded bacterial responses. Opposed effects with regard to these interactions were also established. This should be regarded in further studies.

Several authors assume that endophytic bacteria are considered to be a subset of the bacteria community in soil or rhizosphere (Seghers *et al.*, 2004, Gottel *et al.*, 2011). Some of the bacteria in the rhizosphere or soil have developed mechanisms to penetrate and colonize plant tissues (Quadt-Hallmann *et al.*, 1997, Reinhold-Hurek & Hurek, 1998). Thus, it is not surprising that closest relatives of some of the recovered endophytic bacteria in the present study are of soil or rhizosphere origin. This might explain why bacteria in the endosphere as well as bacteria in the rhizosphere were influenced by different mowing frequencies and fertilization application.

Understanding the influence of different management regimes on plant-associated bacteria is of great importance for predicting future changes in bacterial community composition under different grassland management regimes. This study paved the way for better understanding of bacterial community composition in the rhizosphere and endosphere by integrating different parameters and by co-analyzing both the endophytic and the soil bacterial communities.

CHAPTER E

GENERAL REFERENCES

Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* 59: 143-169.

Araujo WL, Marcon J, Maccheroni W, Jr., Van Elsas JD, Van Vuurde JW, et al. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and Environmental Microbiology* 68: 4906-4914.

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS & Davies WJ (1999). Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. *Functional Ecology* 13: 650-660.

Bardgett RD, Leemans KD, Cook R, Hobbs PJ (1997). Seasonality of the soil biota of grazed and ungrazed hill grasslands. *Soil Biology and Biochemistry* 29: 1285-1294.

Beattie G (2006). Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: Gnanamanickam S (ed). *Plant-Associated Bacteria*. Springer Netherlands. pp 1-56.

Benizri E & Amiaud B (2005). Relationship between plants and soil microbial communities in fertilized grasslands. *Soil Biology and Biochemistry* 37: 2055-2064.

Carlier L, Rotar I, Vlahova M, Vidican R (2009). Importance and functions of grasslands. *Notulae Botanicae, Horti Agrobotanici, Cluj-Napoca* 37: 25-30.

Clegg CD, Lovell RD, Hobbs PJ (2003). The impact of grassland management regime on the community structure of selected bacterial groups in soils. *FEMS Microbiology Ecology* 43: 263-270.

Compant S, Clément C, Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry* 42: 669-678.

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71: 4951-4959.

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

Denef K, Roobroeck D, Manimel Wadu MC, Lootens P & Boeckx P (2009). Microbial community composition and rhizodeposit-carbon assimilation in differently managed temperate grassland soils. *Soil Biology and Biochemistry* 41: 144-153.

Denton CS, Bardgett RD, Cook R & Hobbs PJ (1998). Low amounts of root herbivory positively influence the rhizosphere microbial community in a temperate grassland soil. *Soil Biology and Biochemistry* 31: 155-165.

Doi T, Abe J, Shiotsu F, Morita S (2011). Study on rhizosphere bacterial community in lowland rice grown with organic fertilizers by using PCR-denaturing gradient gel electrophoresis. *Plant Root* 5: 5-16.

Dunfield KE & Germida JJ (2003). Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). *Applied and Environmental Microbiology* 69: 7310-7318.

El Fantroussi S, Verschuere L, Verstraete W & Top EM (1999). Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology* 65: 982-988.

EUROSTAT http://epp.eurostat.ec.europa.eu/statistics_explained/index.php/Agri-environmental_statistics.

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA & Knight R (2011). Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *The ISME Journal* 6: 1007-1017.

Fuentes-Ramírez LE, Caballero-Mellado J, Sepúlveda J & Martínez-Romero E (1999). Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. *FEMS Microbiology Ecology* 29: 117-128.

Garbeva P, Elsas JD & Veen JA (2008). Rhizosphere microbial community and its response to plant species and soil history. *Plant and Soil* 302: 19-32.

Garbeva P, Overbeek LS, Vuurde JW, Elsas JD (2001). Analysis of Endophytic Bacterial Communities of Potato by Plating and Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA Based PCR Fragments. *Microbial Ecology* 41: 369-383.

Gibon A (2005). Managing grassland for production, the environment and the landscape. Challenges at the farm and the landscape level. *Livestock Production Science* 96: 11-31.

Gottel NR, Castro HF, Kerley M*, et al.* (2011). Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* **77**: 5934-5944.

Habekost M, Eisenhauer N, Scheu S, Steinbeiss S, Weigelt A & Gleixner G (2008). Seasonal changes in the soil microbial community in a grassland plant diversity gradient four years after establishment. *Soil Biology and Biochemistry* 40: 2588-2595.

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* 43: 895-914.

Hallmann J, Quadt-Hallmann A, Rodríguez-Kábana R, Kloepper JW (1998). Interactions between *Meloidogyne incognita* and endophytic bacteria in cotton and cucumber. *Soil Biology and Biochemistry* 30: 925-937.

Hallmann J, Rodríguez-Kábana R & Kloepper JW (1999). Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biology and Biochemistry* 31: 551-560.

Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD (2012). Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages. *PLoS ONE* 7: e30438.

Holland J (1995). Effects of above-ground herbivory on soil microbial biomass in conventional and no-tillage agroecosystems. *Applied Soil Ecology* 2: 275-279.

Houlden A, Timms-Wilson TM, Day MJ & Bailey MJ (2008). Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. *FEMS Microbiol Ecology* 65: 193-201. England.

Innes L, Hobbs P & Bardgett R (2004). The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. *Biology and Fertility of Soils* 40: 7-13.

Isselstein J, Jeangros B, Pavlu V (2005). Agronomic aspects of biodiversity targeted management of temperate grasslands in Europe – A review. *Agronomy Research* 3: 139-151.

Katznelson H, Lochhead AG, Timonin MI (1948). Soil microorganisms and the rhizosphere. *The Botanical Review* 14: 543-586.

Kent AD, Triplett EW (2002). Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Reviews in Microbiology* 56: 211-236.

Keuter A, Hoeft I, Veldkamp E, Corre M (2013). Nitrogen response efficiency of a managed and phytodiverse temperate grassland. *Plant and Soil* 364: 193-206.

Kielak A, Pijl AS, van Veen JA & Kowalchuk GA (2008). Phylogenetic diversity of Acidobacteria in a former agricultural soil. *The ISME Journal* 3: 378-382.

Kloepper J, Rodríguez-Kábana R, McInroy J, Young R (1992). Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: Identification by fatty acid analysis and frequency of biological control activity. *Plant and Soil* 139: 75-84.

Kloepper JW, Rodríguez-Kábana R, Zehnder AW, Murphy JF, Sikora E, Fernández C (1999). Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. *Australasian Plant Pathology* 28: 21-26.

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PG & van Veen JA (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek* 81: 509-520.

Kowalchuk GA, Yergeau E, Leveau JH, Sessitsch A, Bailey M (2010). Plant-associated Microbial Communities. *Environmental molecular microbiology*: 131-148.

Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, et al. (2004). Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology* 6: 1244-1251.

Li R, Khafipour E, Krause DO, Entz MH, de Kievit TR, Fernando WGD (2012). Pyrosequencing Reveals the Influence of Organic and Conventional Farming Systems on Bacterial Communities. *PLoS ONE* 7: e51897.

McInroy JA & Kloepper JW (1995). Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Canadian Journal of Microbiology* 41: 895-901.

Nacke H, Thürmer A, Wollherr A, Will C, Hodac L, Herold N *et al* (2011). Pyrosequencing-Based Assessment of Bacterial Community Structure Along Different Management Types in German Forest and Grassland Soils. *PLoS ONE* 6: e17000.

Patra AK, Abbadie L, Clays-Josserand A*, et al.* (2006). Effects of management regime and plant species on the enzyme activity and genetic. *Environmental Microbiology* 8: 1005-1016.

Petersen U, Wrage N, Köhler L, Leuschner C, Isselstein J (2012). Manipulating the species composition of permanent grasslands – A new approach to biodiversity experiments. *Basic and Applied Ecology* 13: 1-9.

Plantureux S, Peeters A, McCracken D (2005). Biodiversity in intensive grasslands: effect of management, improvement and challenges. *Agronomy Research* 3: 153-164.

Quadt-Hallmann A, Kloepper JW, Benhamou N (1997). Bacterial endophytes in cotton: mechanisms of entering the plant. *Canadian Journal of Microbiology* 43: 577-582.

Raaijmakers J, Paulitz T, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* 321: 341-361.

Reinhold-Hurek B & Hurek T (1998). Life in grasses: diazotrophic endophytes. *Trends in Microbiology* 6: 139-144.

Rosenblueth M, Martinez-Romero E (2004). *Rhizobium etli* maize populations and their competitiveness for root colonization. *Archives of Microbiology* 181: 337-344.

Rosenblueth M, Martinez-Romero E (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions* 19: 827-837.

Seghers D, Wittebolle L, Top EM, Verstraete W & Siciliano SD (2004). Impact of agricultural practices on the *Zea mays* L. endophytic community. *Applied and Environmental Microbiology* 70: 1475-1482.

Shafiani S, Malik A (2003). Tolerance of pesticides and antibiotic resistance in bacteria isolated from wastewater-irrigated soil. *World Journal of Microbiology and Biotechnology* 19: 897-901.

Siddiqui I, Shaukat S (2003). Endophytic bacteria: prospects and opportunities for the biological control of plant-parasitic nematodes. *Nematologia Mediterranea* 31: 111-120.

Singh BK, Munro S, Potts JM & Millard P (2007). Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Applied Soil Ecology* 36: 147-155.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H & Berg G (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* 67: 4742-4751.

Sørensen J (1997). The rhizosphere as a habitat for soil microorganisms. *Modern soil microbiology*. 21-45.

Steenwerth KL, Jackson LE, Calderón FJ, Stromberg MR, Scow KM (2002). Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. *Soil Biology and Biochemistry* 34: 1599-1611.

Stoate C, Boatman ND, Borralho RJ, Carvalho CR, de Snoo GR, Eden P (2001). Ecological impacts of arable intensification in Europe. *Journal of Environmental Management* 63: 337-365.

Stoltzfus JR, So R, Malarvithi PP, Ladha JK, de Bruijn FJ (1997). Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant and Soil* 194: 25-36.

Strobel G, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* 67: 491-502.

Sturz AV, Christie BR, Matheson BG, Nowak J (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils* 25: 13-19.

Sturz AV, Nowak J (2000). Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Applied Soil Ecology* 15: 183-190.

Sun L, Qiu F, Zhang X, Dai X, Dong X, Song W (2008). Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microbial Ecology* 55: 415-424.

Tan Z, Hurek T & Reinhold-Hurek B (2003). Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. *Environmental microbiology* 5: 1009-1015.

Techau M, Bjørnlund L & Christensen S (2004). Simulated herbivory effects on rhizosphere organisms in pea (*Pisum sativum*) depended on phosphate. *Plant and Soil* 264: 185-194.

Tscharntke T, Klein AM, Kruess A, Steffan-Dewenter I, Thies C (2005). Landscape perspectives on agricultural intensification and biodiversity – ecosystem service management. *Ecology Letters* ***8:*** 857-874.

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P et al. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology* 68: 2198-2208.

APPENDIX

PUBLICATIONS

PEER-REVIEWED ARTICLES

- Wemheuer B, Wemheuer F, Daniel R. RNA-Based Assessment of Diversity and Composition of Active Archaeal Communities in the German Bight. Archaea, vol. 2012, Article ID 695826, 8 pages, 2012. doi:10.1155/2012/695826.

- Wemheuer B, Taube R, Akyol P, Wemheuer F, Daniel R. Microbial diversity and biochemical potential encoded by thermal spring metagenomes derived from the Kamchatka peninsula. Archaea, vol. 2013: Article ID 136714, 13 pages, 2013. doi:10.1155/2013/136714.

TALKS AT CONFERENCES

- Wemheuer F., Wemheuer, B., Kretzschmar, D., Daniel, R., Vidal S. (2013): Multitrophic interaction between microorganisms, plant and herbivores: Does fertilizing, mowing or herbivory on plants alter the microbial community diversity in the rhizosphere? Talk. “Annual Conference of the German Association for General and Applied Microbiology (VAAM)”, Bremen (10.-13.03.2013).

POSTERS AT CONFERENCES

- Wemheuer, F., Vidal S. (2011): Influence of grassland management intensity on the endophyte diversity in different grass species. International conference “Functions and Services of Biodiversity”, Göttingen, 20.-22.06.2012.

- Wemheuer F., Vidal S. (2011): Influence of grassland management intensity on the fungal endophyte diversity in different grass species. International conference “ProkaGENOMICS”, Göttingen, 18.-21.09.2012.

- Wemheuer F., Vidal S. (2012): Grassland management regimes and grass microbial communities – endophytic species. Workshop “Multitrophic Interactions”, Göttingen, 22.-23.03.2012.

- Wemheuer F., Wemheuer, B., Vidal S. (2012): Multitrophic interaction between microorganisms, plants and herbivores: Does fertilizing, mowing or feeding on plants alter the microbial community composition in the rhizosphere? “International Symposium on Microbial Ecology”, Copenhagen, 19.-24.08.2012.

CURRICULUM VITAE

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WORK EXPERIENCE

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THESIS DECLARATIONS (EIDESSTATTLICHE ERKLÄRUNGEN)

Declaration of the author's own contribution to manuscript with multiple authors

The chapter B contains a series of manuscripts that have or will be submitted to peer-reviewed journals. I am the main author of these manuscripts.

I have personally collected, investigate, and analyzed the data for all manuscripts with one exception. Daniel Kretzschmar helped me with the laboratory work in year 2012 during my pregnancy. I developed the main ideas presented in these manuscripts, and written all manuscripts. Furthermore, I made the tables, figures, and appendices.

The working group of Prof. Dr. Rolf Daniel, especially Bernd Wemheuer, helped with the analysis of the next generation sequencing data. My supervisors gave helpful remarks for the discussion and writing of all manuscripts. The other co-author Sarah Herzog contributed to the discussion of the results.

Declaration plagiarism

I hereby confirm that I have written this doctoral thesis independently. I have not used other sources or facilities others than the ones mentioned in the chapters. Moreover, I have not used unauthorized assistance and that I have not submitted this thesis previously in any form for another degree at any institution or university.

Franziska Wemheuer

Göttingen, May 2013