

Impact of Oxygen and Pesticides on Microbial Cellulose Degradation in Aerated Agricultural Soils: A Microscaled Analysis of Processes and Prokaryotic Populations

Dissertation

To obtain the Academic Degree
Doctor rerum naturalium
(Dr. rer. nat.)

Submitted to the Faculty of Biology, Chemistry and Earth Sciences
of the University of Bayreuth

by
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Bayreuth, November 2011

The present study was prepared between November 2007 and November 2011 at the Department for Ecological Microbiology at the University of Bayreuth under the direction of Prof. Harold L. Drake.

The work was financially supported by the Deutsche Forschungsgemeinschaft (DFG Ko2912/3-1) and the University of Bayreuth.

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat).

Dissertation eingereicht: 11.11.2011
Zulassung durch die Prüfungskommission: 28.02.2012
Wissenschaftliches Kolloquium: 24.04.2012

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ABBREVIATIONS

\bar{x}	arithmetic mean
S_x	standard error
S_y	error propagation
%	percent
∅	diameter
°C	degree centigrade
μl	microliter
μm	micrometer
μM	micromolar; micromole per liter
μmol	micromole
^{12}C	most common carbon isotope on earth
^{13}C	heavy isotope of the element carbon
α	1. Bunsen solubility coefficient; 2. significance level (t-Test)
A_{260}, A_{280}	adsorption at 260 nm, and 280 nm
Ad	fill up to
AOA	ammonium-oxidizing archaea
AOB	ammonium-oxidizing bacteria
APS	ammonium persulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	1. carbon; 2. coverage
c	concentration
cDNA	copy deoxyribonucleic acid
C_{DNAStd}	concentration of standard DNA
cm	centimeter
CMC	carboxymethyl-cellulose
conc.	concentration
CsTFA	caesium trifluoroacetate
C_T	threshold cycle
CTAB	cetyl trimethylammonium bromide
C_{Ts}	number of target sequences
d	day
ddH ₂ O	deionized double-distilled water
DEPC-H ₂ O	diethylpyrocarbonate-treated water
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
DNR	dissimilatory nitrate reduction
DNRA	dissimilatory nitrate reduction to ammonium
dNTP	deoxyribonucleotide
dsDNA	double-stranded DNA
DT ₅₀	half-life time in days at 20°C
DTT	dithiothreitol

DW	dry weight
E	amplification efficiency
e.g.	<i>exempli gratia</i> (Latin); 'for example'
EC	Enzyme Commission number
EDTA	Ethylenediaminetetraacetate
E_h	redox potential
EPA	United States Environmental Protection Agency
et al.	<i>et alii</i> (Latin), 'and others'
F	forward primer
FAO	Food and Agriculture Organization of the United Nations
f_d	degree of freedom (t-Test)
FID	flame ionization detector
FISH	fluorescence in situ hybridization
FW	fresh weight
g	gram
g	gravitational acceleration
GC	gas chromatography
h	hours
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; buffer
HPLC	high performance liquid chromatography
i.e.	<i>id est</i> (Latin); 'that is'
INHIBCORR	assay for correction of inhibition (qPCR)
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
IRD	Infra-red dye
IUPAC	International Union of Pure and Applied Chemistry
K	degree Kelvin
kb	kilobase
l	liter
<i>lacZ</i>	gene encoding the β -galactosidase
LB	Lurani-Bertani; culture medium
lg	decadic logarithm
Lt ₂	Clay loam (German soil classification)
MCPA	2-methyl-4-chlorophenoxyacetic acid
MCS	multiple cloning site
m_{DW}	dry weight of soil
m_{FW}	fresh weight of soil
mg	milligram
min	minute
ml	milliliter
mM	millimolar; millimole per liter
mmol	millimole
mol	mole
MOPS	3-(N-morpholino)propanesulfonic acid
<i>MspI</i>	Restriction endonuclease isolated from <i>Mooraxella</i> sp. (ATCC 4967)
mV	millivolt
MW	molecular weight
MW _{bp}	Molecular weight of one base pair in double-stranded DNA

N	1. nitrogen; 2. total number of analysed sequences
n	1. amount of substance, 2. number of replicates/considered values
n.a.	non-applicable
n.i.	1. not identified 2. no inhibition
N_A	Avogadro constant
NaCl	sodium chloride
NaOH	sodium hydroxide
N_{bases}	length of amplicon
NCBI	National Center for Biotechnology Information
n_{gas}	amount of gas in the gaseous phase
$n_{\text{gel,p}}$	amount of physically dissolved gas
n_{ges}	total amount of gas
$n_{\text{ges,c}}$	amount of chemically dissolved gas
nm	nanometer
nM	nanomolar; nanomole per liter
no.	number
OD ₆₆₀	optical density at 660 nm
OTU	operational taxonomic unit
p_1	standardized air pressure
PAGE	Polyacrylamide gel
p_{akt}	actual air pressure
PCR	polymerase chain reaction
PCR-H ₂ O	particle-free autoclaved water
PEG	polyethylene glycol
Pg	petagramm
pg	picogramm
pH	the negative decimal logarithm of the hydrogen ion activity in a solution
<i>pheA</i>	gene encoding a phenol hydrolyase
pK_a	acid dissociation constant
PLFA	phospholipid fatty acids
<i>pmoA</i>	gene encoding the particulate methane monooxygenase
ppm	parts per million
$p_{\text{ü}}$	overpressure in incubation flasks
qPCR	quantitative polymerase chain reaction
R	reverse primer
R^2	stability index
RDP	Ribosomal Database Project
RID	refractive index detector
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription PCR
S	standard deviation
Seq.	sequence
S_i	number of expected OTUs
SIP	stable isotope probing

SOC	super optimal broth; medium
sp.	species
SQ	starting quantity
t	1. ton (=1,000 kg); 2. value that implies statistical significance
T ₁	standardized temperature
TAE	tris-aceate-EDTA; buffer
T _{akt}	actual temperature
Taq	thermostable DNA polymerase isolated from <i>Thermus aquaticus</i>
TaqI	Restriction endonuclease isolated from <i>Thermus aquaticus</i>
TBE	tris-borat-EDTA; buffer
TCD	thermal conductivity detector
TEMED	N,N,N,N-tetramethylethyldiame
temp.	temperature
T _m	basic melting temperature
tRF	terminal restriction fragment
tRFLP	terminal restriction fragment length polymorphism
Tris	tris(hydroxymethyl)-aminomethane
U	unit
UFZ	Helmholtz-Center for Environmental Research
UV	ultraviolet
v	variance
V	1. volume; 2. volt
v/v	volume per volume
V ₁	standardized molar gas volume
V _{akt,mol}	molar volume of gas under actual conditions
V _{fl}	volume of liquid phase
V _{gas}	volume of the gas phase
VWD	variable wavelength detector
w/v	weight per volume
W _g	gravimetric water content
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

1.1. Carbon Flow through Terrestrial Ecosystems

Conversion of biomass to carbon dioxide (CO₂) and methane (CH₄) in terrestrial ecosystems is a substantial part of global carbon cycling (Amundson 2001; Bernstein et al. 2007). The global carbon cycle is a long-term balance between atmospheric carbon, and terrestrial and aquatic carbon sinks and carbon sources (Dumonceaux 2005; Falkowski et al. 2000). Atmospheric carbon occurs primarily as carbon dioxide and its concentration increased during the last two centuries to 385 ppm (Lal 2008). The total amount of carbon in the atmosphere is about 780 Pg (Figure 1) and increases about 4.1 Pg year⁻¹ due to anthropogenic activities (Amundson 2001; Bernstein et al. 2007; Lal 2008).

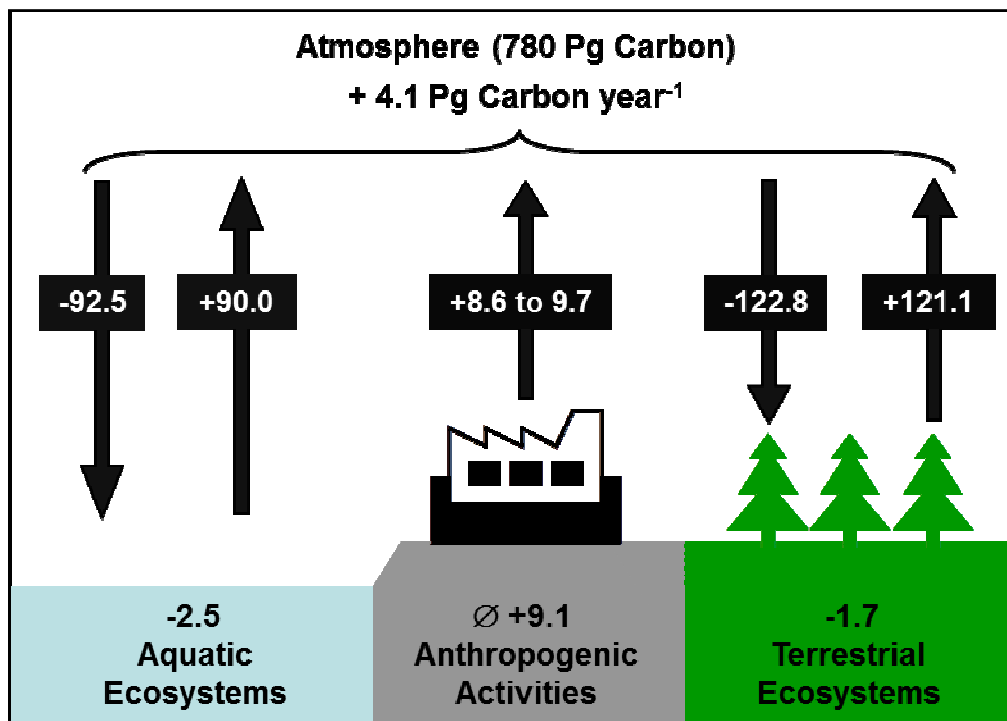


Figure 1. Schematic scheme of the global carbon flow. Global carbon pools and exchange of carbon with the atmosphere are displayed. Numbers represent estimated amounts of carbon in Pg year⁻¹. Negative numbers indicate carbon consumed by the ecosystem type. Positive numbers indicate carbon that is released to the atmosphere. Values are according to Lal 2008.

Anthropogenic activities like fossil fuel burning (7.5 Pg carbon year⁻¹) and intensive land use (~1.6 Pg carbon year⁻¹) emit more than 9 Pg carbon year⁻¹ (Figure 1; Lal 2008). Oceans that take up 2.5 Pg of anthropogenic carbon dioxide and terrestrial ecosystems are important sinks for atmospheric carbon dioxide (Schimel 1995). It is estimated that ~120 Pg of carbon year⁻¹ are fixed by plants in the form of atmospheric carbon dioxide and stored in plant biomass (Dumonceaux 2005). However, 2.6 Pg more carbon is fixed by unknown terrestrial sinks (Lal 2008). Autotrophic respiration by plants (~60 Pg year⁻¹) and disturbances, e.g., wild fires,

contribute to the release of large amounts of carbon dioxide back into the atmosphere (Falkowski et al. 2000; Lal 2008). Another 60 Pg carbon year⁻¹ is returned to the atmosphere by biological decomposition of plant biomass. Soil organisms (e.g., bacteria, fungi, protists) aerobically and anaerobically metabolize plant-derived carbon, produce carbon dioxide, and close the global carbon cycle (Dumonceanu 2005; Lal 2008). An important characteristic of soil is that it has the capacity to store large amounts of atmospheric carbon dioxide. Atmospheric carbon dioxide is photosynthetically fixed and put into the soil as recalcitrant plant-derived compounds (Amundson 2001; Lal 2008). Soils take up 1.7 Pg of anthropogenic carbon year⁻¹ (Figure 1), making them to a key ecosystem concerning the global carbon budget. The amount of carbon that is stored in soil is determined by a lot of diverse factors, e.g., climate, topographic position, temperature, potential soil biota, or human activities (Amundson 2001). In contrast, a significant part of carbon dioxide that is returned back into the atmosphere primarily depends on the activity of soil organisms that decompose plant matter and plant-derived carbon sources (Falkowski et al. 2000). Conversion of natural forests, grasslands, and wetlands to agricultural ecosystems (i) decreases the amount of organic carbon that is stored in soil and (ii) increases the amount of carbon dioxide that is released into the atmosphere (Schimel 1995; Schlesinger and Andrews 2000). Especially tillage by crop farming facilitates carbon loss from soil by improving conditions for microbial decomposition of organic carbon. Crop farming leads to better soil aeration and higher soil temperatures, and simultaneously reduces the input of plant material into soil compared to the native vegetation (Amundson 2001; Schlesinger and Andrews 2000). Fertilization or use of pesticides in agriculture influences carbon flow in agricultural soils (Lal 2008; Schlesinger and Andrews 2000) and might also impact on soil organisms that are involved in the decomposition of plant-derived organic matter. Hence, soil organisms that decompose plants are key players in the global carbon cycle since they drive a substantial part of carbon flow through terrestrial ecosystems.

1.2. Cellulose – a Major Polysaccharide in Soils

Biological decomposition of plant material to carbon dioxide is a complex process that starts at structural polymers. The lignocellulose complex forms the cell wall of plants and is composed of lignin, cellulose, hemicelluloses, and other polymers (Chang 2007; Kumar et al. 2008a; Malherbe and Cloete 2002). Lignocellulose is the most abundant and renewable energy source on earth. Terrestrial plants produce as much biomass as equivalent to two-thirds of the world's energy requirement per year (Demain et al. 2005). The polysaccharide cellulose is the major component of lignocellulose and constitutes 35 – 50% of the dry weight of plants or litter from deciduous and coniferous trees (Kögel-Knabner 2002; Lynd et al. 2002). Dependent on the stage of growth, cotton can be composed of up to 95% of cellulose (Abidi et al. 2010). However, minor amounts of cellulose are found in cell wall polymers of certain bacteria, fungi, slime molds, and amoebae (Coughlan 1985; Lynd et al. 2002; Tomme et al. 1995). It is estimated that cellulose is produced on earth by photosynthesis at least at a rate of $10^9 - 10^{10}$ t year⁻¹ and that a similar amount is degraded by cellulolytic, i.e., cellulose-degrading, and saccharolytic organisms, i.e., organisms that metabolize products of cellulose hydrolysis like cellobiose or glucose (Coughlan 1985; Henrissat 1994). Structure and hydrolysis of cellulose has been extensively studied due to the increasing interests of biotechnologists and others for industrial use of this biopolymer (e.g., Bayer et al.

2008; Beguin and Aubert 1994; Berghem et al. 1975; Dubos 1928; Gardner and Blackwell 1974a; Gardner and Blackwell 1974b). However, relatively little is known about the diversity and function of organisms that might participate in the degradation of cellulose in soil.

1.2.1. Chemical Structure of Cellulose and its Enzymatic Hydrolysis

Cellulose is a polysaccharide composed of β -D-glucose units that are linked by β -1,4-glycosidic bonds (Gardner and Blackwell 1974b; Kumar et al. 2008a). These linear cellulose chains have a variable degree of polymerization of up to 15,000 glucose units in cotton (Coughlan 1985). Cellulose chains are linked by intermolecular hydrogen bonds and form thereby crystalline cellulose (microfibrils) (Figure 2; O'Sullivan 1997). The degree of crystallinity is variable (Teeri 1997). The polysaccharide cellulose is insoluble and cannot be directly assimilated by cellulolytic organisms (Lynd et al. 2002). Cellulolytic organisms excrete hydrolytic enzymes, the so called cellulases, outside their cell wall, because they are unable to transport cellulose across the cell membrane (Wilson 2008). The tight structure of crystalline cellulose is broken up these cellulases what releases soluble saccharides (e.g., cellodextrins, cellobiose, and glucose) that are transported into the cell and further metabolized (Desvaux 2005b; Wilson 2008).

Fungal and bacterial cellulases are well studied and have been classified based on structural properties and their specific enzymatic activities (Henrissat et al. 1998; Rabinovich et al. 2002). Cellulases attack crystalline cellulose in amorphous regions, i.e., regions that are not strongly stabilized by intramolecular hydrogen bonds (Beguin and Aubert 1994; Lynd et al. 2002; O'Sullivan 1997). Cellulases may be released as non-complexed (1.2.1.1) or as complexed enzyme systems (1.2.1.2) (Bayer and Lamed 1992; Bayer et al. 1998b; Desvaux 2005b). Non-complexed cellulases are primarily synthesized by aerobes, whereas complexed cellulase systems are synthesised by cellulolytic anaerobes (e.g., by *Clostridium thermocellum*; Bayer et al. 1998a; Demain et al. 2005; Wilson 2009a). In general, a tight attachment of cellulolytic organisms to the substrate is required to efficiently hydrolyze cellulose and optimize the consumption of released saccharides (Beguin and Aubert 1994).

Three major types of cellulases are known (according to Lynd et al. 2002): (i) endoglucanases (or 1,4- β -D-4-glucan glucanohydrolases; EC 3.2.1.4), (ii) exoglucanases, including cellobiohydrolases (or 1,4- β -D glucan-4-glucan cellobiohydrolases; EC 3.2.1.91) and cellodextrinases (or 1,4- β -D-glucan glucanohydrolases; EC 3.2.1.74), and (iii) β -glucosidases (or β -glucoside glucohydrolases; EC 3.2.1.21). β -glucosidases act as extracellular and intracellular enzymes (Bedino et al. 1985; Inglin et al. 1980; Meyer and Canevascini 1981; Mihoc and Kluepfel 1990). Cellulases are distinguished from other glycoside hydrolases by their ability to (i) hydrolyze β -1,4-glycosidic bonds between glucosyl residues and (ii) to act not exclusively at chain ends (Bayer et al. 1998a; Lynd et al. 2002).

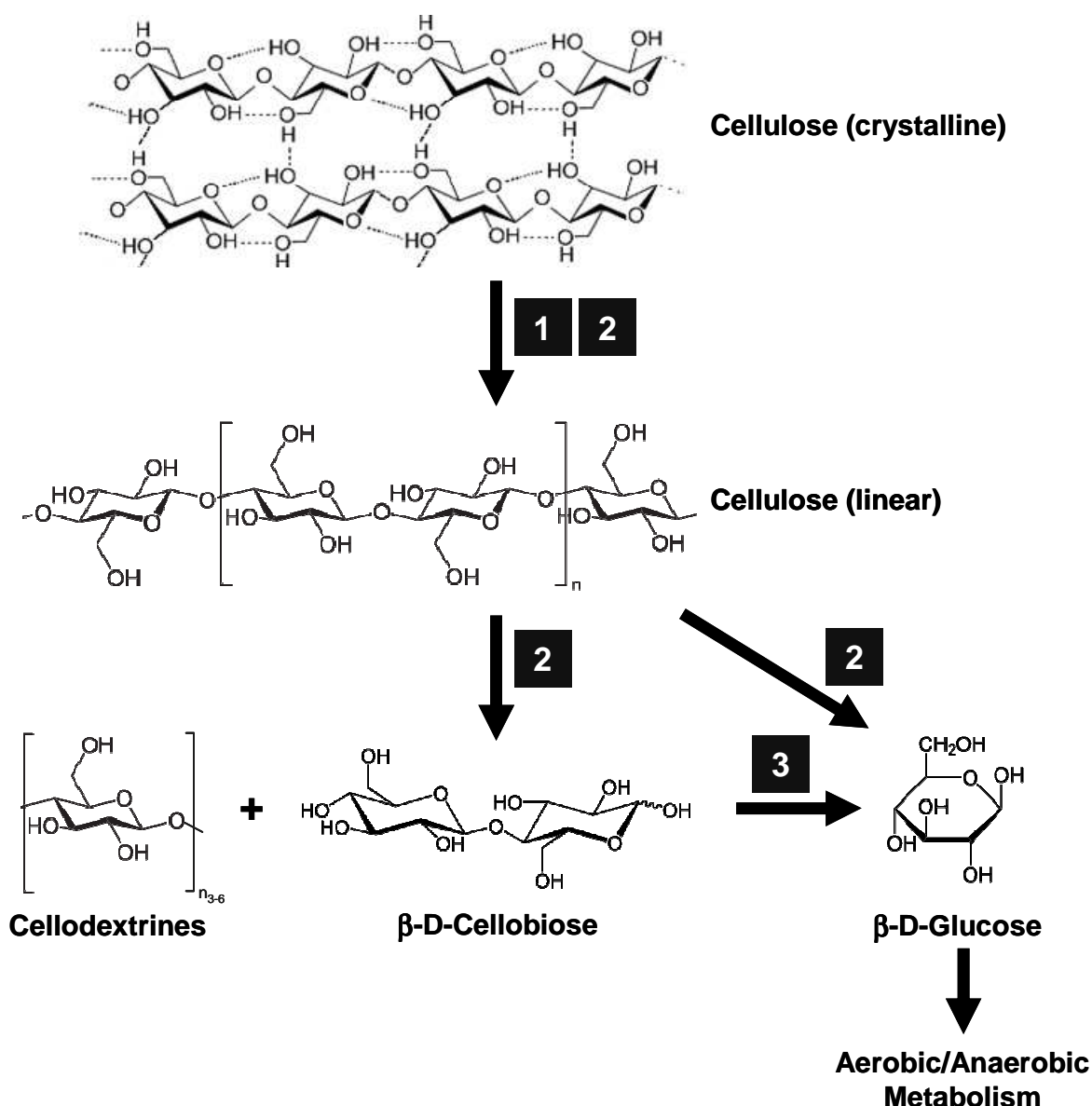


Figure 2. Enzymatic hydrolysis of crystalline cellulose. Composite scheme based on Bayer et al. 1998a; Bisaria and Ghose 1981; Lynd et al. 2002; Teeri 1997. Enzyme groups and the step of hydrolysis at which they attack the compounds are indicated by numbers: 1, Endoglucanases (EC 3.2.1.4); 2, Exoglucanases, including cellobiohydrolases (EC 3.2.1.91) and cellodextrinase (EC 3.2.1.74); 3, β -glucosidases (EC 3.2.1.21). Dotted lines in crystalline cellulose indicate molecular hydrogen bonds.

Crystalline cellulose is attacked by endoglucanases (**1**) at internal amorphous regions and randomly cut into linear cellulose chains of various lengths, leading to new chain ends (Figure 2). Exoglucanases (**2**) can also act on crystalline cellulose fibres, presumably peeling off cellulose chains (Figure 2). Exoglucanases (**2**) attack cellulose chains in a processive manner on reducing and non-reducing ends yielding cellodextrins, cellobiose, and glucose; with cellobiose and glucose being the major products (Figure 2). Soluble cellodextrins and cellobiose are cut by β -glucosidases (**3**) into β -D-glucose (Figure 2). All types of enzymes act synergistically (e.g., Beguin and Aubert 1994; Beguin 1990; Henrissat 1994; Mansfield et al. 1999; Rabinovich et al. 2002). The saccharides cellobiose and glucose are metabolized by a broad

diversity of aerobic and anaerobic cellulolytic and saccharolytic organisms (Bayer et al. 2006; Lynd et al. 2002; Figure 3 and Figure 4).

Cellulase enzyme systems of bacteria and fungi function similar and follow the same complex induction-repression mechanisms (Beguin and Aubert 1994; Coughlan 1991; Kumar et al. 2008a; Lynd et al. 2002). Cellulases and β -glucosidases are expressed constitutively at low levels, and excessive production is regulated by further mechanisms (Beguin and Aubert 1994; Busto et al. 1995; Lynd et al. 2002). In many organisms, cellulase biosynthesis is induced in the presence of cellulose or soluble hydrolysis products like cellobiose or cellodextrins. All known cellulase systems are additionally repressed in the presence of low molecular weight carbon compounds such as glucose that are more easily metabolized than cellulose (Beguin and Aubert 1994; Stewart and Leatherwood 1976). Cellobiose is the main intermediate of the entire cellulose hydrolysis process (Corazza et al. 2005). It is not only an inducer of cellulase expression, but also a regulator for endo- and exoglucanase activity. Both types of glucanases are inhibited by cellobiose upon a certain concentration (e.g., at 0.6 mM cellobiose in cultures of *C. thermocellum*; Beguin and Aubert 1994; Berghem et al. 1975; Gong et al. 1977; Zhang and Lynd 2005). β -glucosidases hydrolyze cellobiose into two glucose molecules to prevent product inhibition of glucanases. Hence, the cleavage of cellobiose is often the rate-limiting step in cellulose hydrolysis (Bhatia et al. 2002; Corazza et al. 2005). β -glucosidase activity in some cellulolytic fungi is regulated via substrate and product inhibition (e.g. in *Trichoderma viridae*; Bhatia et al. 2002; Hong et al. 1981). In contrast, the activity of β -glucosidases in the cellulolytic bacterium *C. thermocellum* is not influenced by glucose (Katayeva et al. 1992). Several other substrate- and enzyme-related factors, such as degree of substrate polymerisation/crystallinity or accessible surface area, are also determinants of enzymatic cellulose degradation (Kumar et al. 2008a; Mansfield et al. 1999).

1.2.1.1. Non-Complexed Enzyme Systems

Non-complexed cellulase systems are mainly found in aerobic organisms and are well studied in fungi (e.g., *Trichoderma* sp.; Ilmen et al. 1997; Mandels and Reese 1957; Yang et al. 2004; Zhang and Lynd 2006) and bacteria (e.g., *Cellulomonas* sp. and *Thermobifida* sp.; Chaudhary et al. 1997, Irwin et al. 1993; Irwin et al. 1998; Lamed et al. 1987; Warren 1996). Each organism that uses non-complexed enzyme systems secretes a set of individual cellulases into the environment. The fungus *Trichoderma reesei* excretes a set of five endoglucanases, two exoglucanases, and two β -glucosidases, whereas species of *Cellulomonas* excrete at least six endoglucanases and one exoglucanase (Lynd et al. 2002). A general feature of most cellulases is a modular structure including non-catalytic cellulose-binding domains (also termed as carbohydrate-binding modules) and catalytic domains (Bayer et al. 1998a; Doi 2008; Lynd et al. 2002; Wilson 2009a). The carbohydrate-binding modules mediate attachment of the enzyme to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate, and to initiate processing of exoglucanases (Lynd et al. 2002; Teeri et al. 1998).

1.2.1.2. Complexed Enzyme Systems (Cellulosomes)

Complexed enzyme systems are typically known from organisms living in anaerobic environments (Bayer et al. 2004; Lynd et al. 2002). Cellulosomes are large multienzyme complexes with numerous subunits tightly linked (Bayer and Lamed 1986; Mayer et al. 1987). All components form a huge protein complex, also called protobozyme (Bayer et al. 1994). Cellulosomes are flexible enough to bind to the cell wall of the cellulolytic organism and simultaneously to the surface of cellulose. They mediate cellular adhesion and form a corridor between the cell and the substrate by changing their conformation after binding (Bayer et al. 1994; Lynd et al. 2002). The component that distinguishes cellulosomes from free non-complex enzyme systems is a non-catalytic high molecular weight scaffolding protein that replaces the carbohydrate-binding module (Bayer et al. 1994; Bayer et al. 2004; Doi 2008; Wilson 2009a). The cellulosome allows optimal synergism between the excreted cellulases and also minimizes the distance over which hydrolysis products must diffuse. This facilitates efficient uptake of enzymatically released saccharides by the organism (Bayer et al. 1994; Lynd et al. 2002; Schwarz 2001). Cellulosomes are well studied in anaerobic bacteria (e.g., in *Clostridia* sp.; Desvaux 2005a; Petitdemange et al. 1984; Schwarz 2001; Zhang and Lynd 2005), but cellulosome-like structures are also known for some anaerobic fungi that are found in the digestion tract of ruminants (Doi 2008; Lynd et al. 2002; Orpin 1975; Rabinovich et al. 2002).

1.2.1.3. Extracellular Cellulose Disrupting Complex in Gram-negative Bacteria

Recently, a third mechanism for cellulose hydrolysis was proposed for two gram-negative bacteria (Wilson 2008; Wilson 2009b). The aerobe *Cytophaga hutchinsonii* and the anaerobe *Fibrobacter succinogenes* neither use complexed nor non-complexed enzyme systems for the degradation of cellulose. Both bacteria do not encode processive endo- and exocellulases as are typically for other cellulolytic organisms (Wilson 2008). *C. hutchinsonii* exclusively encodes for non-processive endoglucanases and lacks carbohydrate-binding modules as well as scaffolding proteins (Xie et al. 2007). *F. succinogenes* does also not encode for known processive cellulases or scaffolding proteins (Jun et al. 2007; Malburg et al. 1997). However, both organisms can grow efficiently on cellulose (Fields et al. 2000; Nakagawa and Yamasato 1996). It is suggested that both organisms use a similar, but not identical extracellular cellulose disrupting complex that is bound to the outer cell membrane. This complex removes linear cellulose chains from crystalline cellulose and transports them through the outer membrane into the periplasmic space. In the periplasm these chains are hydrolysed by endoglucanases and released saccharides are transported through the plasma membrane into the cell for further metabolism (Wilson 2009a).

1.2.2. Biological Degradation of Cellulose in Soils

Cellulose is a major source of carbon for soil microbial communities, and is degraded under oxic and anoxic conditions (Lynd et al. 2002; Bayer et al. 2006). Decomposition of complex plant biomass is catalyzed by various aerobic and anaerobic organisms (i.e., microorganisms capable of anaerobiosis, which includes obligate anaerobes and facultative aerobes) of the domains *Bacteria* and *Eukarya* (Baldrian and Valaskova 2008; Lynd et al. 2002). For decades, it has been hypothesized that degradation of cellulose is exclusively carried out by fungi and

bacteria, but it appears that some animals produce their own cellulases (1.3.1), which differ substantially from those of their indigenous microbial community (Watanabe and Tokuda 2001; Watanabe and Tokuda 2010).

Cellulolytic and saccharolytic microorganisms inhabit similar ecological niches, and compete for carbon and energy sources. However, it was shown that aerobic degradation of cellulose by pure cultures of *Cellulomonas flavigena* is less efficient than degradation by mixed cultures of cellulolytic and saccharolytic species (Patel and Vaughn 1973). Species of *Clostridium* degrade cellulose much more efficiently in the presence of other non-cellulolytic bacteria than in pure culture (Enebo 1949; Kato et al. 2004). The saccharolytic partners improve cellulose degradation by efficient removal of potentially inhibiting hydrolysis products, e.g. cellobiose and glucose. These observations indicate that interactions of taxa with different substrate spectra might be important for the efficient degradation of cellulose (1.2.1).

More than 90% of the global amount of cellulose is degraded in well aerated agricultural, grassland or forest soils (Bastian et al. 2009; Kurka 2001; Leschine 1995; Lynd et al. 2002; Vinten et al. 2002). Aerobic fungi and bacteria are the main degraders of cellulosic biomass in soil (Bastian et al. 2009; de Boer et al. 2005), and hydrolysis of cellulose mainly yields cellobiose and glucose (1.2.1). These saccharides are taken up for energy conservation and carbon assimilation, which allows for cell growth and formation of biomass (Desvaux 2006). The main end products of cellulose decomposition under oxic conditions are carbon dioxide and water (H₂O; Bayer et al. 2006; Beguin and Aubert 1994; Schmidt and Ruschmeyer 1958).

Anoxic environments in which anaerobic degradation of cellulose occurs are mainly found in the digestive tract of animals (Leschine 1995; Watanabe and Tokuda 2001), deep soil sediments (Leschine 1995), composts (de Bertoldi et al. 1983), and water-saturated environments such as freshwater, marine and estuarine sediments, flooded soils, or wetlands (Conrad 1996; Glissmann and Conrad 2000; Leschine 1995). Agricultural soils (except for flooded rice fields) are usually water-unsaturated and aerated. Nonetheless, anoxic microzones with low concentrations of oxygen (O₂) and low redox potential occur (1.6) in which anaerobic microbial activities can take place (Küsel and Drake 1995; Pett-Ridge and Firestone 2005; Picek et al. 2000; Totsche et al. 2010). Less studies investigated the degradation of crystalline cellulose and plant-derived carbon under anoxic conditions in aerated soils (Leschine 1995; Lynd et al. 2002), although anaerobic cellulolytic *Bacteria* are frequently isolated from various types of aerated soil (e.g., An et al. 2005; Ohmiya et al. 2005; Ozkan et al. 2001; Rampersad et al. 1998). Aerobic fungi are also important degraders of cellulosic biomass under oxic conditions, but fungal species are apparently not important cellulose degraders in soil under anoxic conditions (Baldrian and Valaskova 2008; de Boer et al. 2005). Thus, prokaryotes, i.e., *Bacteria*, represent the majority of known microorganisms that are primarily responsible for the degradation of cellulose under anoxic conditions (Hu and vanBruggen 1997)

In anoxic microzones, cellulose and cellulose-derived saccharides are decomposed by obligate anaerobic and facultative aerobic bacteria via an intermediary ecosystem metabolism (Drake et al. 2009; Lynd et al. 2002; Wüst et al. 2009). In permanently flooded or wetland soils, fermentation of soluble saccharides by primary fermenters (e.g., *Enterobacteriaceae*; Degelmann et al. 2009a) yields fatty acids, alcohols, molecular hydrogen, and carbon dioxide. These compounds serve as substrates for secondary fermenters, acetogens, and methanogens (Drake et al. 2009; Drake et al. 2008; Hamberger et al. 2008; McInerney and Struchtemeyer 2008;

Schink and Stams 2006; Wüst et al. 2009) making carbon dioxide and methane the terminal end products (e.g., Beguin and Aubert 1994; Desvaux 2006; Drake et al. 2009; Westermann 1996). In marine systems, sulfate-reducing bacteria out-compete methanogens for molecular hydrogen, resulting in H_2S as a major product of the anaerobic degradation of cellulose (Ljungdahl and Eriksson 1985; Peck and Odom 1981). The anaerobic degradation of saccharides in transient anoxic microzones of aerated soils (Sexstone et al. 1985; Drake et al. 2006; Zausig et al. 1993) may be similar to that of flooded and wetland soils, but not identical (Figure 3; Conrad 1996; Glissmann and Conrad 2000).

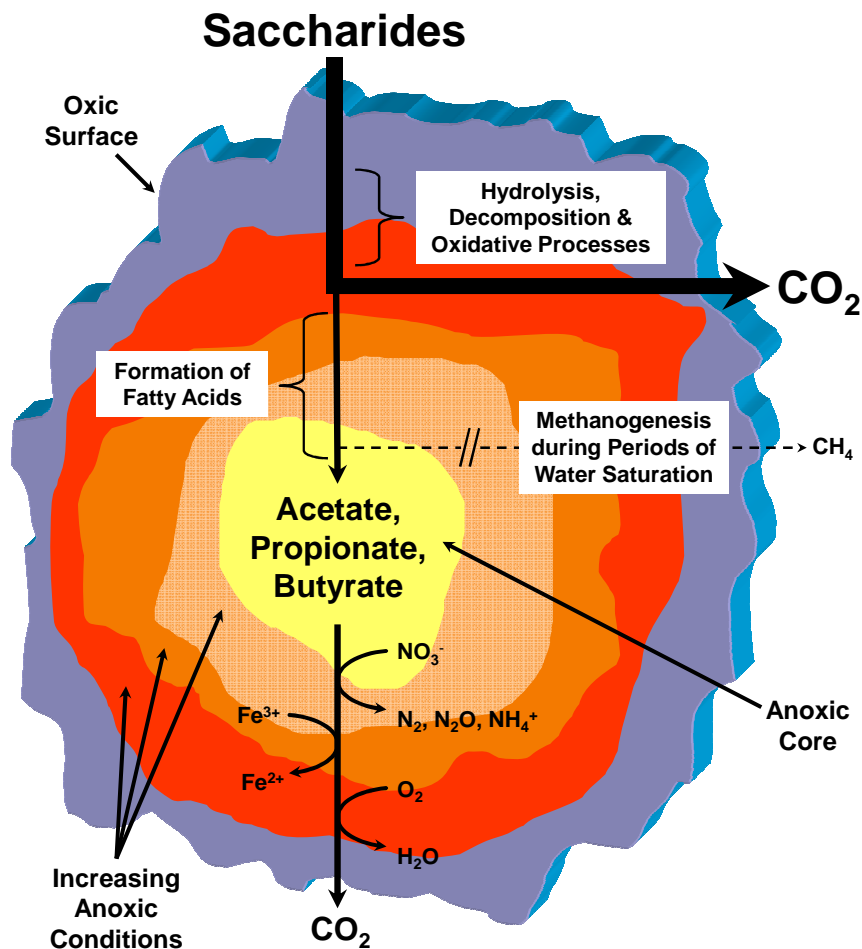


Figure 3. Cross-section of a soil aggregate in aerated soil showing a hypothetical model of a possible intermediary ecosystem metabolism during the anaerobic degradation of cellulose-derived saccharides. The initial hydrolysis of cellulose is not shown and precedes the processes illustrated in the model. Model according to Drake et al. 2006 and Wüst et al. 2009.

Cellulose-derived saccharides are oxidized to carbon dioxide in well-aerated zones, e.g., the oxic aggregate surface (Figure 3). Increasing anoxic conditions from the surface to the inside facilitate the anaerobic degradation of saccharides by fermentation and anaerobic respiration. The oxidation of transient organic intermediates such as acetate, propionate, or butyrate is coupled to alternative redox processes, i.e., denitrification, the reduction of ferric iron (Fe^{3+}), or the reduction of O_2 when conditions become oxic (Küsel et al. 2002). Methane is usually not formed, making methanogenesis not a major route of carbon mineralization in aerated soils (e.g. Küsel and Drake 1994; Küsel and Drake 1995).

1.3. Diversity of Cellulose-Degrading Organisms

Cellulolytic organisms are ubiquitous in nature, and are found in the domains *Bacteria* (kingdom *Prokarya*), *Archaea* (kingdom *Prokarya*), and within fungi and protists of the kingdom *Eukarya* (e.g., Bayer et al. 2006; Lynd et al. 2002; Margulis et al. 1990). Numerous cellulolytic bacteria and fungi are available in culture and their physiology is often well studied (Bagnara et al. 1985; Baldrian and Valaskova 2008; Herculano et al. 2011; Petitdemange et al. 1984). Although interactions of cellulolytic and saccharolytic *Bacteria* have been investigated (Dumova and Kruglov 2009; Enebo 1949; Kato et al. 2004; Patel and Vaughn 1973), their role in agricultural ecosystems during the degradation of cellulose is still widely unresolved. Due to the low cultivability of *Prokaryotes* and the high abundance of cellulose in soils (Bayer and Lamed 1992; Zhang and Xu 2008), it is reasonable that a large uncultured diversity of soil *Bacteria* contributes to the degradation of cellulose in agricultural soils.

1.3.1. *Eukaryotes*

Cellulose utilization is distributed across the entire kingdom of fungi. The anaerobic degradation of cellulose is restricted to the primitive, protist-like group of *Chytridiomycetes*. These fungi are well known for their ability to degrade cellulose in the gastrointestinal tract of ruminants and other non-ruminant herbivores (e.g., Bauchop 1979; Gordon and Phillips 1998). They are obligate anaerobes, and conserve energy from the hydrolysis of xylan and cellulose by fermentation of these carbohydrates (Gordon and Phillips 1998; Orpin 1994). Anaerobic cellulolytic species of fungi are found in the genera *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpimomyces*, and *Anaeromyces* (Ho and Abdullah 1999; Ho et al. 2000; Teunissen and DenCamp 1993). They were numerically isolated from the intestine of ruminants, dried and fresh feces, but not from soil (Gordon and Phillips 1998). The capability to degrade cellulose under oxic conditions is well represented among the remaining subdivision of aerobic fungi. A large number of cellulolytic species of *Zygomycetes* (e.g., *Mucor*; Botha et al. 1997), *Ascomycetes* (e.g., *Chaetotium*; Manoliu et al. 2005), *Basidiomycetes* (e.g., *Phanerochaete*; Eriksson 1978), and *Deuteromycetes* (e.g., *Aspergillus*; Herculano et al. 2011) have been isolated. These organisms have received considerable study with respect to their wood-degrading capability and their cellulolytic enzymes for industrial use (Cohen et al. 2002; Schmidt 2007).

Other organisms that degrade cellulose are known from protists (Watanabe and Tokuda 2001; Watanabe and Tokuda 2010). Cellulolytic protists are members of the microbial community of ruminants (e.g., the ciliate *Eudiplodinium maggii*; Coleman 1978), where they ingest cellulosic material directly or graze on bacteria that

themselves utilize cellulose (Orpin 1984). The soil-inhabiting slime mold *Dictyostelium discoideum* also produces cellulolytic enzymes, but their major function is likely related to the maturation of spores (Beguin and Aubert 1994; Blume and Ennis 1991). A wide range of invertebrates also produces endogenous cellulases (Watanabe and Tokuda 2001; Watanabe and Tokuda 2010). It was shown that some termites themselves can produce cellulase components (Watanabe et al. 1997), although they harbor cellulolytic protists and bacteria in their gut (Breznak and Brune 1994). Their role of these endogenous cellulases in the nutrition of animals is largely unresolved and their contribution to the degradation of cellulose in soil is likely of minor importance.

1.3.2. *Prokaryotes*

The capability to degrade cellulose is widespread among numerous taxa of the domain *Bacteria* (Table 1), but only one described isolate of *Archaea* is able to utilize cellulose. *Desulfurococcus fermentans* is an extreme thermophilic and cellulolytic species that was isolated from a hot spring and grows optimally above 80°C (Perevalova et al. 2005). Furthermore, an archaeal thermophilic enrichment can also grow on crystalline cellulose (Graham et al. 2011; Robb et al. 2011). It can be suggested that thermophilic *Archaea* do not contribute to the degradation of cellulose in temperated agricultural soils. No cellulolytic *Archaeon* of temperate or cold soils has been discovered to date.

Most cultivated cellulolytic aerobic bacteria belong to the phylum *Actinobacteria* (Lynd et al. 2002). Cellulolytic *Actinobacteria* were frequently isolated from soil or rotten biomass (Abdulla and El-Shatoury 2007; An et al. 2005; Eriskon 1949; Kang et al. 2007). Cellulolytic *Bacteroidetes* of the genus *Cytophaga* are known since decades (Stanier 1942; Stanier 1947), and other cellulolytic representatives with an aerobic metabolism are found in *Alpha*-, *Beta*-, *Delta*-, and *Gammaproteobacteria*, and *Firmicutes* (Table 1). Most anaerobic cellulolytic bacteria belong to the phylum *Firmicutes* (Lynd et al. 2002). These bacteria are well known for and often isolated from the rumen (Kamra 2005; Montgomery et al. 1988), and other predominantly anaerobic environments like waste composts or sludge (Ohmiya et al. 2005; Patel et al. 1980; Petitdemange et al. 1984). Species of the *Clostridiales* (phylum *Firmicutes*) have been intensively investigated in regard to their biochemical pathways and genetics of the anaerobic degradation of cellulose (Demain et al. 2005; Desvaux 2005a; Ng and Zeikus 1982). The phyla *Acidobacteria*, *Bacteroidetes*, *Fibrobacteres*, *Spirochaetes*, and *Thermotogae* (Table 1) also contain some anaerobic cellulolytic species.

Table 1. List of bacterial phyla including cultured aerobic and anaerobic cellulolytic species.

Phylum	Representative species	Reference
Aerobes		
<i>Actinobacteria</i>	<i>Actinotalea fermentans</i> ^a	Yi et al. 2007
<i>Alphaproteobacteria</i>	<i>Sphingomonas echinoides</i>	Wenzel et al. 2002
<i>Bacteroidetes</i>	<i>Cytophaga hutchinsonii</i>	Nakagawa and Yamasato 1996
<i>Betaproteobacteria</i>	<i>Jeongeupia naejangsanensis</i>	Yoon et al. 2010
<i>Deltaproteobacteria</i>	<i>Sorangium cellulosum</i>	Lampky 1971
<i>Gammaproteobacteria</i>	<i>Saacharophagus degradans</i>	Ekborg et al. 2005
<i>Firmicutes</i>	<i>Bacillus circulans</i>	Kim 1995
Anaerobes		
<i>Acidobacteria</i>	<i>Telmatobacter bravus</i>	Pankratov et al. 2011
<i>Bacteroidetes</i>	<i>Bacteroides cellulosolvens</i>	Murray et al. 1984
<i>Firmicutes</i>	<i>Clostridium cellulolyticum</i>	Petitdemange et al. 1984
<i>Fibrobacteres</i>	<i>Fibrobacter succinogenes</i>	Montgomery et al. 1988
<i>Spirochaetes</i>	<i>Spirochaeta thermophila</i>	Aksenova et al. 1992
<i>Thermotogae</i>	<i>Fervidobacterium islandicum</i>	Huber et al. 1990

^a *Cellulomonas* and *Actinotalea* (both family *Cellulomonadaceae*) are the only known genera that contain facultative aerobic cellulolytic species.

Based on analysis of pure cultures it appears that assimilation of crystalline cellulose by bacteria is primarily catalysed by either strict aerobic or strict anaerobic taxa (Lynd et al. 2002). Although some facultative aerobes produce cellulase genes under both oxic and anoxic conditions (Bakalidou et al. 2002; Pason et al. 2006; Schumann et al. 2001; Yi et al. 2007), *Cellulomonas* and *Actinotalea* are the only known genera that contain facultative aerobic species capable of aerobic and anaerobic cellulose utilization (Bagnara et al. 1985; Bagnara et al. 1987; Clemmer and Tseng 1986; Reguera and Leschine 2001; Yi et al. 2007). Few studies investigated the cellulose-degrading community in soils. *Actinobacteria*, *Bacteroidetes*, and *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria* have been identified as potential degraders of cellulose under oxic conditions in agricultural soils (Bernard et al. 2007; Haichar et al. 2007), whereas members of the *Firmicutes* were the main degraders of cellulose in municipal solid waste samples under anoxic conditions (Li et al. 2009). It is not clear which factors are responsible for the strict differentiation of aerobic and anaerobic cellulolytic taxa under such contrasting availabilities of O₂ (Lynd et al. 2002).

1.4. Detection of Cellulose-Degrading Organisms

Over decades, cellulose-degrading organisms have been studied without the use of gene-based molecular techniques. The lack of such tools in microbiology during the first three quarters of the last century resulted in the discovery of cellulolytic bacteria and fungi from various habitats by cultivation (e.g., Enebo 1949; Hofsten et al. 1971, Lieckfeldt et al. 2000; Orpin 1975; Petitdemange et al. 1984). However, it remains difficult to resolve the diversity of a functional group that catalyzes the same biological process in complex habitats by cultivation. With advances in molecular techniques, more and more studies investigated the cellulolytic soil community by gene-based approaches (e.g., Bastias et al. 2009; Haichar et al. 2007; Li et al. 2009). A suitable molecular attempt to analyse the diversity of a functional groups is the use of structural gene markers. Structural genes are genes that encode for process-related key enzymes. For example, the *pmoA* gene encodes for subunits of the particular methane-monooxygenase, a key enzyme of methanotrophs (Kolb et al. 2003; Murrell and Radajewski 2000). This is not possible for cellulose-degrading bacterial and fungal communities. Although cellulases are the key enzymes of cellulolytic microorganisms, cellulases exhibit a high genetic and structural heterogeneity (Bayer et al. 2006; Lynd et al. 2002). The development of primers for broad-based detection of cellulase genes is not possible. Hence, more general gene-markers have to be used to assess structure and diversity of cellulolytic communities in the environment. 16S/18S rRNA primers target the cellulose-degrading community, but they cannot resolve which of the detected species is cellulolytic, saccharolytic, or not involved in the degradation of cellulose. To date there is a large gap of understanding between the diversity of soil organisms and their functions in agricultural ecosystems. Nevertheless, the combination of 16S/18S rRNA gene-based approaches with other methods, e.g., stable isotope probing (SIP, Manefield et al. 2002a; Manefield et al. 2002b; Radajewski et al. 2000; Radajewski et al. 2003), might allow for identification of microorganisms that assimilate cellulose-derived carbon in a complex environment such as soil (Bastias et al. 2009; Haichar et al. 2007).

1.5. Cellulose-Degrading Microorganisms in Soil Have Similar Ecological Niches

Soil contains numerous spatialized microenvironments/habitats that are sufficient for the existence of different microbial phenotypes (e.g., aerobic or anaerobic, cellulolytic or saccharolytic organisms). The sum of abiotic and biotic factors that are present in a certain habitat defines the limits in which ecological niches of microorganisms can be realized (Hutchinson 1957). Ecological niches of microbial species in a habitat can be similar, but a single niche cannot be simultaneously occupied by two different species (Hutchinson 1957). Cellulose in nature is primarily available in the form of wood or dead plant material and is present as lignocellulose complex that has high lignin content (Lynd et al. 2002). Cellulose fibers are not available for organisms as long as they are embedded in lignin. Despite the ability of various microorganisms to degrade cellulose, some aerobic white rot, brown rot, and soft rot fungi are the only organisms that are capable to attack lignin (Cullen and Kersten 2004; de Boer et al. 2005; Eriksson et al. 1990; Hammel 1997). Growth of bacteria on intermediates derived from the degradation of lignin has been observed (Cespedes et al. 1997; Falcon et al. 1995; Vicuna et al. 1993), but direct degradation of lignin by bacteria seems to be negligible in terrestrial environments

(Kirk and Farrell 1987; Tuomela et al. 2000). Hence, fungi are important organisms for the degradation of plant-derived lignocellulose and cellulose in soil. Lignin-degrading fungi initially attack plant matter with their lignolytic enzymes leading to uncovered embedded cellulose (Cullen and Kersten 2004; Hammel 1997; Van der Waal et al. 2007). Exposed cellulose fibers subsequently serve as substrate for cellulolytic organisms (Lynd et al. 2002). Although cellulolytic fungi and bacteria share the same habitat and catalyse the same ecosystem function, i.e., the degradation of plant-derived cellulose, they occupy different ecological niches and are favoured under different environmental conditions. For example, acidic pH (Matthies et al. 1997; Rousk et al. 2010), low deposition of nitrogen (Zechmeister-Boltenstern et al. 2011), and well-aerated conditions may promote growth of fungi, whereas bacteria may be promoted by a more neutral pH (> 5) or higher concentrations of nitrogen (Hu and vanBruggen 1997; McCarthy 1987). Anoxic conditions may also select for cellulolytic bacteria, since anaerobic cellulolytic fungi are relevant in the rumen, but not in soil (de Boer et al. 2005; Kamra 2005; Orpin 1984). However, less is known about interactions of organisms that are functionally redundant, i.e., that contribute to the same ecological function in an agricultural soil, and that occur in close proximity in contrasting microenvironments. The current understanding of cellulose degradation in soils can be summarized in the following conceptual model (Figure 4; Bayer et al. 1994; Bayer et al. 2006).

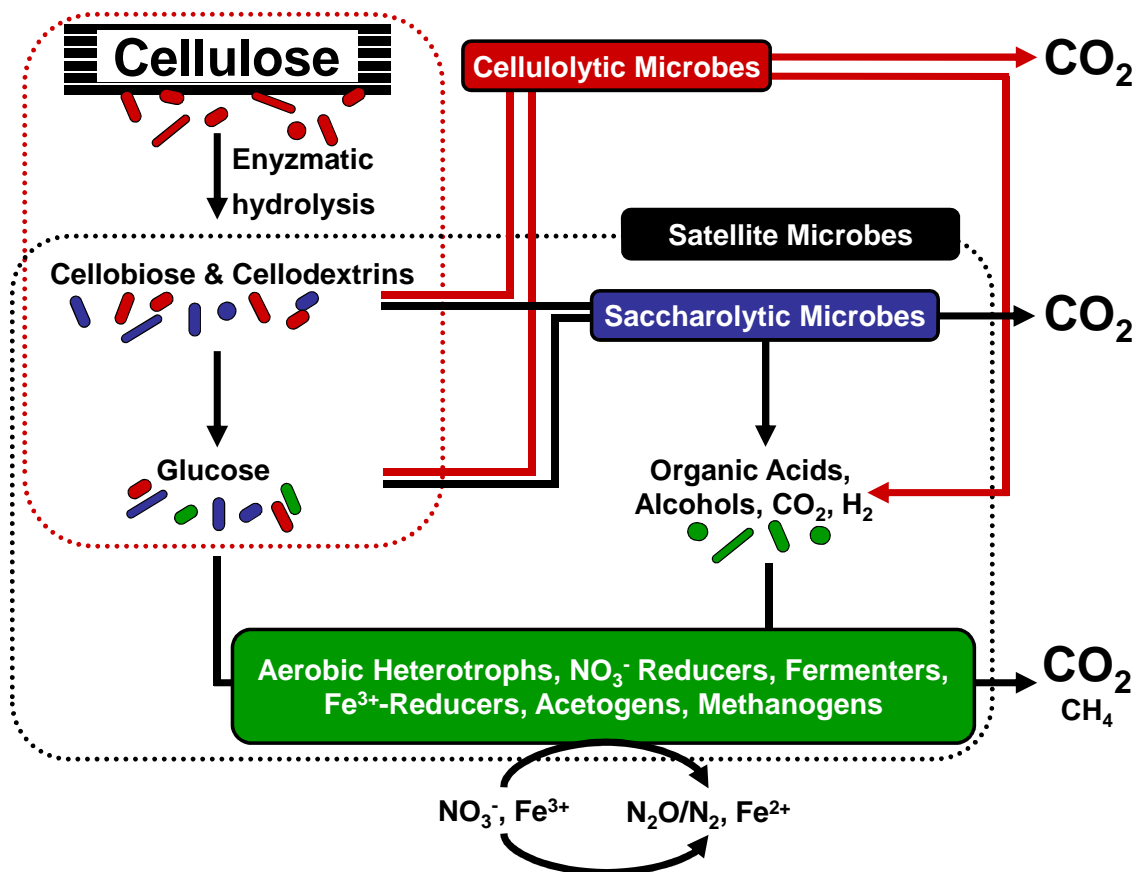


Figure 4. Model of interactions between cellulolytic and non-cellulolytic satellite microorganisms in oxic soils (according to Bayer et al. 1994; Bayer et al. 2006). The smaller font size of CH_4 indicates that it is a minor product under most *in situ* conditions.

Cellulolytic organisms attack cellulose with their extracellular glycosidic enzyme systems, and produce cellobiose and glucose (Figure 4). Most of the hydrolysis products are directly assimilated by the cellulolytic microbe, but some products might be released into the environment (Bayer et al. 1994; Bayer et al. 2006). The removal of free cellulose-derived sugars by saccharolytic satellite species keeps these sugars at low non-inhibitory concentrations (Bayer et al. 1994; Doi 2008), and hence, prevents cellobiose- and glucose-induced inhibition of endo- and exocellulases of cellulolytic organisms (1.2.1). Products of saccharide degradation include fatty acids, alcohols, carbon dioxide, and molecular hydrogen (Figure 4). These compounds are further assimilated and dissimilated by other non-saccharolytic satellite microorganisms (e.g., aerobic heterotrophs, nitrate reducers, secondary fermenters, iron reducers, acetogens, or methanogens) to (i) carbon dioxide under oxic and (ii) carbon dioxide and methane under anoxic conditions (1.2.2) concomitantly to the reduction of alternative electron acceptors such as nitrate (NO_3^-) or ferric iron. In summary, the degradation of cellulose in a cellulosic environment is always facilitated by a complex microbial community consisting of cellulolytic and non-cellulolytic satellite organisms.

1.6. Environmental and Anthropogenic Factors Influence Bacterial Cellulose Degradation in Agricultural Soil

The degradation of cellulose is an important community function in soil ecosystems, and is catalyzed by anaerobic and aerobic saccharide-degrading microorganisms (1.2.2). The bacterial community that is responsible for this process is confronted with changing environmental conditions, because agricultural soil is normally a water-unsaturated, aerated, and highly structured environment that comprises dynamic physicochemical gradients at biogeochemical interfaces (Or et al. 2007; Six et al. 2000; Totsche et al. 2010). The distribution of oxic and anoxic microzones is variable, spatially heterogeneous, and dependent on soil moisture and soil aggregate size (Dassonville et al. 2004; Or et al. 2007; Zausig et al. 1993). Oxic and anoxic compartments co-occur on a small scale in the same soil. Aerobic and anaerobic microorganisms exist in these oxic and anoxic microzones, and their metabolic activity is determined by various chemical and physical parameters (Brune et al. 2000; Or et al. 2007; Totsche et al. 2010; Yadav and Malanson 2007).

1.6.1. Changing Availability of O_2 Induces Redox Changes

In water-unsaturated soils, soil moisture content is an important soil parameter that is closely related to the availability of O_2 and that can change rapidly (Denef et al. 2001; Mansfeldt 2004; Vorenhout et al. 2004). The consumption of O_2 exceeds the diffusion of O_2 at the interface of gas-filled and water-saturated pores, and promotes the formation of anoxic microzones (e.g., inside soil aggregates, Figure. 3; Brune et al. 2000; Dassonville et al. 2004; Greenwood 1961). A sudden increase of soil moisture, e.g. after a rain event, can increase the size of these anoxic microenvironments (Denef et al. 2001; Mansfeldt 2004; Vorenhout et al. 2004). O_2 is the terminal electron acceptor for aerobic respiration (Brune et al. 2000) and the depletion of O_2 facilitates short term shifts from oxidizing to reducing conditions (Denef et al. 2001; Mansfeldt 2004; Vorenhout et al. 2004). The availability of O_2 has a tremendous impact on the redox potential of soil and also on the energy metabolism/catabolism of the microorganisms (Brune et al. 2000). It is measured as a potential difference (E_h in [mV]) between an inert electrode and a reference cell

(Bohn 1971; Fiedler et al. 2007). The sequential reduction of electron acceptors in aerated soil under flooded conditions correlates with the thermodynamic theory (Peters and Conrad 1996) that predicts that the reduction of electron acceptors should be sequential in the order of their standard redox potentials (Zehnder and Stumm 1988). Thus, the redox potential of soil is (i) a suitable measure for the presence of oxidizing and reducing conditions, and (ii) for prediction of redox reactions and microbial activities like denitrification, ferric iron reduction, fermentations, and methanogenesis. After the depletion of the preferred electron acceptor O_2 alternative electron acceptors, such as nitrate, manganese (Mn^{2+}), or ferric iron, are utilized by anaerobic microorganisms, and redox potentials of soil may drop to negative values as consequence of the depletion of oxidized compounds (Dassonville et al. 2004; Peters and Conrad 1996). The rate at which redox potentials drop is dependent in part on the availability of organic carbon that serves as energy and electron source for microbial metabolism, and on the concentration of alternative electron acceptors (Bohn 1971; Brune et al. 2000; Dassonville et al. 2004). The redox potential may return to more oxidizing conditions due to the increased availability of O_2 and the activation of aerobic microorganisms after a decrease of soil moisture by drought. Thus, fluctuation of the availability of O_2 (as a direct consequence of soil moisture content) is an environmental factor that impacts on the activity of microbial taxa in agricultural soils (Brune et al. 2000; Donnelly et al. 1990; Devevre and Horwath 2000; Jacobson and Jacobson 1998; Kremen et al. 2005). However, it is not known, how single members of the cellulose-degrading community respond to fluctuations in the availability of O_2 .

1.6.2. Pesticides Influence Biological Processes

Microorganisms in agricultural soils are not only challenged by fluctuating availabilities of O_2 , but can also be influenced by anthropogenic disturbances (Cox et al. 1996; Johnsen et al. 2001; Katayama and Kuwatsuka 1991; Porter and Hayden 2002). The increased usage of herbicides, fungicides, and insecticides over the past 70 years has resulted in an accumulation of pesticide residues in terrestrial and aquatic environments (Akerblom 2004; Hiller et al. 2008; Thorstensen et al. 2001). Pesticides are subjected to microbial degradation in soils (Chowdhury et al. 2008; Gonzalez et al. 2006; Müller et al. 2001; Sukul and Spittler 2001), but their degradation is usually slow compared to natural organic compounds and occurs primarily under oxic conditions (Harrison et al. 1998; Knauber et al. 2000). Pesticide degradation is generally slower under anoxic conditions (Harrison et al. 1998; Knauber et al. 2000). Some pesticides and break down products thereof are highly mobile and bioavailable in soils (Johnsen et al. 2001; Spliid et al. 2006), and thus, might impact on soil microorganisms (Chowdhury et al. 2008; Gevao et al. 2000; Katayama and Kuwatsuka 1991; Katayama et al. 1992; Wainwright 1978). The herbicide Bentazon inhibits bacterial growth in pure culture and microbial activity in soils (Cernakova et al. 1991; Galhano et al. 2009; Marsh et al. 1978). Bentazon-treated soils display decreased capacities for nitrogen mineralization and dinitrogen (N_2) fixation (Galhano et al. 2009; Marsh et al. 1978). Similarly, the herbicide MCPA (4-Chloro-2-methylphenoxy acetic acid) reduces growth and activity of yeast, *Pseudomonas putida*, and *Vibrio fischeri* in pure culture (Ahtiainen et al. 2003; Cabral et al. 2003), but its toxicity to soil prokaryotes and associated processes is unresolved. Nonylphenol suppresses cellular respiratory activity of bacteria (Hseu 2006, Okai et al. 2000b). The fungicide Chlorothalonil reduces cellulose decomposition in aerated soils due to inhibition of cellulolytic fungi and *Bacteria*

(Katayama and Kuwatsuka 1991; Katayama et al. 1992; Suyama et al. 1993a; Suyama et al. 1993b). β -glucosidase (1.2.1) activity is inhibited by the fungicide Metalaxyl in soil (Monkiedje and Spiteller 2002; Sukul 2006). Thus, the application of pesticides might alter cellulose-dependent carbon flow in soil and might influence associated microbial taxa.

1.7. Hypotheses and Objectives

The biological degradation of plant-derived cellulose is an important function of agricultural ecosystems. The activity of cellulose-utilizing microbes in agricultural soil is determined by several natural and anthropogenic factors, two important ones being the distribution of O_2 and the increasing usage of pesticides. Both factors might influence the cellulose-degrading community, and might alter the degradation rates of cellulose in soil. The process of cellulose degradation has been intensively studied (Bayer et al. 2006; Berghem et al. 1975; Henrissat 1994), but it is widely unresolved if the capacity of soil to degrade cellulose is also a stable trait of agricultural ecosystems when the availability of O_2 changes rapidly. Furthermore, it is unresolved how pesticides affect trophic interactions in cellulolytic communities, and thereby, influence cellulose mineralization. There is still a large unknown diversity of cellulose-degrading microorganisms, and few studies evaluated the activity and distribution of cellulolytic bacteria in agricultural soil (Bernard et al. 2007; Haichar et al. 2007). To understand carbon flow through cellulolytic soil communities, it is necessary to identify active microorganisms and to characterize their metabolic response to fluctuations of regulating environmental factors. Thus, the following hypotheses were addressed:

Hypothesis 1: Different cellulolytic and saccharolytic taxa catalyze the degradation of cellulose in response on the presence or absence of O_2 .

Hypothesis 2: The selective activation of functionally redundant cellulolytic and saccharolytic taxa yields a stable degradation of cellulose under fluctuating availabilities of O_2 .

Hypothesis 3: Pesticides impact on the metabolic activity of cellulose-degrading microbial taxa and decrease cellulose degradation.

Objectives of the doctoral thesis were to analyze the aerobic and anaerobic dissimilation and assimilation of [^{13}C]-cellulose, [^{13}C]-cellobiose, and [^{13}C]-glucose in oxic and anoxic soil microcosms of an agricultural soil. Both prokaryotic and eukaryotic taxa that incorporated [^{13}C]-labeled carbon in their nucleic acids were identified by RNA-based stable isotope probing (1.4), with the primary focus being on the prokaryotic community. Subsequently, taxon-specific 16S rRNA primers were developed to resolve the metabolic response of major cellulolytic and saccharolytic bacterial taxa of the investigated soil (i) to fluctuating availabilities of O_2 using quantitative PCR and (ii) to pesticides during the degradation of cellulose and cellulose-derived saccharides.

2. MATERIAL AND METHODS

2.1. Chemicals, Gases, and Media

Unless otherwise stated chemicals and gases were supplied by Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), BioRad (Richmond, USA), Boehringer (Mannheim, Germany), Carl Roth (Karlsruhe, Germany), Eppendorf (Hamburg, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), and Rießner (Lichtenfels, Germany). Deionised double-distilled water (ddH₂O) was produced with the ultrapure water purification system Seralpur Pro 90 CN (Seral Erich Alhäuser, Ransbach-Baumbach, Germany). PCR-H₂O was prepared by filtration (\varnothing 0.2 μ m) of ddH₂O and autoclaving. RNase/DNase-free water (DEPC-H₂O) was achieved by treating of ddH₂O with 0.1% (v/v) diethylpyrocarbonate (DEPC). After incubation (37°C, 1 hour, slow shaking [300 rpm]) the solution was autoclaved (120°C, 1 bar, 20 minutes) for inactivation of DEPC. Different culture media (Table 2 and Table 3) were used for transformation of competent cells and cultivation of clones. (2.5.12). Composition and preparation as follows:

Table 2. LB medium (Lurani-Bertani medium).

Component	Amount	Final Concentration
Tryptone	10 g	1%
Yeast extract	5 g	0.5%
NaCl	5 g	85 mM
ddH ₂ O	Ad 1,000 ml	-

Tryptone, yeast extract, and NaCl were mixed with 980 ml ddH₂O. The pH was adjusted to 7.0 and ddH₂O was added to a final volume of 1,000 ml. Subsequently, the medium was autoclaved. For the use of LB plates 1.5% (w/v) agar was added to the medium before autoclaving. The medium was poured into sterile plastic petri dishes and stored at 4°C after solidification.

For blue/white-screening of clones (2.5.12.3) LB-plates (LB/Ampicillin/IPTG/X-Gal-plates) were prepared that contained ampicillin, isopropyl- β -D-thiogalactopyranoside (IPTG), and 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Autoclaved LB-medium (Table 2) was cooled down to 50°C and 1 ml ampicillin (100 mg ml⁻¹), 1 ml (IPTG, 0.5 M) and 1.6 ml X-Gal (50 mg ml⁻¹ in N,N'-dimethylformamide) were added. The medium was poured into sterile plastic petri dishes and stored at 4°C after solidification.

Table 3. SOC medium (Super-Optimal-Browth medium).

Component	Amount	Final Concentration
Tryptone	2.0 g	2.0%
Yeast extract	0.5 g	0.5%
NaCl solution (1 M)	1.0 ml	10 mM
KCl solution (1 M)	0.25 ml	2.5 mM
Mg ²⁺ solution (2 M) ^a	1.0 ml	20 mM
Glucose solution (2 M)	1.0 ml	20 mM
ddH ₂ O	Ad 100 ml	-

^a Mg²⁺ solution: 24.6 g MgSO₄, 20.3 g MgCl₂, ad 100 ml.

Tryptone, yeast extract, NaCl, and KCl were mixed with 97 ml ddH₂O and autoclaved. After cool down Mg²⁺ and glucose (both sterile filtered, Ø 0.2 µm) were supplied, and sterile ddH₂O was added to final volume of 100 µl. The medium was again sterile filtered and stored at -20°C.

2.2. Sampling Site and Soil Characteristics

Soil samples were taken from an agricultural soil that was planted with corn and located near the research farm Kloostergut Scheyern, Germany (Table 4). Soil from the upper 20 cm was sampled randomly with a spade and samples were pooled. Soil that was not used immediately was stored at 2°C. Directly before use soil was homogenized by sieving (mesh size: 2 mm, Retsch, Haan, Germany).

Table 4. Soil characteristics^a.

Klostergut Scheyern, Germany	
Coordinates	48°30.0 'N, 11°20.7 'E
Soil type ^b	Dystric Cambisol
Texture (clay : silt : sand) ^c	28 : 33 : 40 (\pm 0 : 0 : 0) \triangleq clay loam (Lt ₂)
Sampling dates	November 2007, April 2008/2010, June 2009
W _g (%)	19.9 \pm 3.6
Total C (%) ^c	1.2 \pm 0.0
Total N (%) ^c	0.2 \pm 0.0
C/N ratio ^c	6.9 \pm 0.1
pH (in water)	6.5 \pm 0.4
pH (in CaCl ₂)	6.0 \pm 0.1
Inorganic ions^d	$\mu\text{mol g}_{\text{soil DW}}^{-1}$
NH ₄ ⁺	2.9 \pm 4.7
NO ₃ ⁻	11.1 \pm 8.9
Total Fe	533.8 \pm 41.0
Total Mn	17.2 \pm 1.5
SO ₄ ²⁻	0.3 \pm 0.2

^a Values are the means of 3 – 21 replicates with standard deviation.

^b According to FAO-classification (Food and Agriculture Organization of the United Nations; Fuka et al. 2008).

^c Department of Soil Ecology, University of Bayreuth, Germany. Lt₂ (German soil classification; Eckelmann 2005).

^d Institute for Central Analytics, University of Bayreuth, Germany.

W_g, Gravimetric water content.

DW, Dry weight.

2.3. Soil Microcosms

Microcosms were prepared as slurries (soil : water, 1 : 2.5) or by using field fresh soil without additional water. Values of substrate-free controls were included in any experiment to determine indigenous carbon turnover and linked processes. Substrate-free controls were used for correction of substrate-supplemented treatments to directly show substrate-related processes. Values obtained in substrate-free controls were subtracted from values in substrate-supplemented treatments at the corresponding time point. Incubations were performed at 15°C or 20°C in the dark.

2.3.1. Treatments for Stable Isotope Probing (SIP)

Stable isotope probing experiments were performed to resolve aerobic and anaerobic dissimilation of three model substrates, and to identify prokaryotes that assimilated substrate-derived carbon (2.5.6). Crystalline cellulose, the disaccharide cellobiose, and the monosaccharide glucose were used in either its [^{12}C]- or [^{13}C]-isotopic form (uniformly labeled; IsoLife, Wageningen, The Netherlands).

Field fresh, sieved soil was mixed with 2.5 volumes of sterile oxic or anoxic double distilled water (ddH_2O) and mixed by extensive manual shaking for 5 minutes. Slurries were placed on ice and flushed with sterile dinitrogen (100%; Rießner, Lichtenfels, Germany) or sterile air for 1 hour. Slurries were homogenized on an end-over-end shaker (Reax 2, Heidolph, Schwabach, Germany) for 1.5 hours at 5°C, divided into 80 ml aliquots, and filled in butyl rubberstoppered 500 ml (anoxic) or 1 l (oxic) flasks (Müller & Krempel, Bülach, Switzerland) with dinitrogen or air as atmosphere. After the addition of substrate (2.3.1.1., 2.3.1.2) incubation was performed at 15°C on an end-over-end shaker for 84 days (cellulose) or 24 days (cellobiose, glucose). Every 7 days cellulose-supplemented treatments were flushed for 15 minutes with sterile dinitrogen or air. Cellobiose- or glucose-supplemented treatments were flushed every 2 days (oxic) or 4 (anoxic) days. Exchange of the headspace was performed to minimize 'cross-labeling' by either the autotrophic or heterotrophic fixation of the [^{13}C]-carbon dioxide formed during the degradation of the primary labeled substrate.

2.3.1.1. Supplementation of Cellulose

0.2 g of [^{13}C]-cellulose or [^{12}C]-cellulose was added once due to the insolubility of this polymer. The amount of supplemented cellulose corresponds to approximately 6.6 mmol of carbon based on the assumption that 1 mol cellulose (independent of degree of polymerization) is equivalent to 1 mol glucose ($\text{MW} = 180 \text{ g mol}^{-1}$). [^{13}C]-treatments were duplicated, [^{12}C]-treatments were not replicated. Endogenous carbon turnover was monitored in unsupplemented oxic and anoxic controls (2.3).

2 ml of the liquid and 0.2 ml of the gaseous phase were taken every 7 days with sterile syringes for analysis of methane, carbon dioxide and molecular hydrogen (2.4.6), soluble organic compounds (2.4.7), inorganic compounds (2.4.10), and pH measurements (2.4.4). Gases were immediately analysed after sampling, followed by the exchange of the headspace. 2 ml of the liquid phase were additionally taken for molecular analyses at day 0, 35, and 70 (2.5), and stored at -80°C until use. In collaboration with Dr. Antonis Chatzinotas (Department of Ecological Microbiology, Helmholtz Center for Environmental Research, UFZ Leipzig, Germany) cDNA (2.5.7) samples of day 0, 35 and 70 derived from oxic and anoxic [^{12}C]- and [^{13}C]-gradients were sent to UFZ Leipzig and used to determine if also eukaryotic organisms (i.e., fungi and protists) were labeled in the experiment.

2.3.1.2. Supplementation of Cellobiose and Glucose

The substrate, i.e., [^{12}C]- or [^{13}C]-cellobiose or [^{12}C]- or [^{13}C]-glucose, was pulsed periodically, i.e., daily in oxic treatments and every two days in anoxic treatments. In total, 0.5 ml of a substrate stock solution (40 mM) were added 12 times. The concentration of glucose and cellobiose (0.25 mM per pulse) was five times greater than maximum concentration detected in soils (Hill et al. 2008; Medeiros et al. 2006), and thus was supplied at amounts that represented a compromise between the low *in situ* concentrations and that needed for effective [^{13}C] incorporation in the rRNA pool. [^{13}C]-supplemented treatments were set up in triplicates, [^{12}C]-supplemented and unsupplemented control treatments (2.3) in duplicates. The total amounts of cellobiose and glucose pulsed were 0.24 mmol, which corresponds to 2.88 and 1.44 mmol of carbon, respectively.

Analogous to cellulose-supplemented treatments (2.3.1.1) methane, carbon dioxide and molecular hydrogen (2.4.6), soluble organic compounds (2.4.7), inorganic compounds (2.4.10), pH measurements (2.4.4), and exchange of the headspace were performed every 2 days (oxic) or 4 days (anoxic). Additionally, 2 ml of the liquid phase were sampled for molecular analyses that were performed at day 0, 6, and 12 in oxic treatments, and at day 0, 4, 20, and 24 in anoxic treatments (2.5).

2.3.2. Treatments in Self-Constructed Incubation Chambers

The effect of periodically changing O_2 availabilities on the degradation of carboxymethyl-cellulose (CMC) and cellobiose was analysed in self-constructed incubation chambers (Figure 5). Previously identified cellulolytic and saccharolytic taxa (2.5.6) were analysed for their metabolic response on the availability of O_2 .

2.3.2.1. Design of Incubation Chambers

Incubation chambers (Figure 5) were constructed in close collaboration with G. Kűfner, and P. Schmidt and colleagues (Wissenschaftliche Werkstätten, University of Bayreuth, Germany). The central element was a plexiglas cylinder (inner Ø : 100 mm; height: 125 mm; wall thickness: 10 mm) that was stuck to a plastic disk (150 mm x 150 mm). The cover plate (Ø : 150 mm) was equipped with a closable sensor port for insertion of pH/redox sensors, two ball valves for gas in- and gas outlet, and an exchangeable septum (Ø : 10 mm, thickness: 3 mm) for sampling of the liquid phase. The cover plate was fixed gastight with four thread-forming screws. The gas inlet valve was linked by a plexiglas tube with the gas distribution plate located at the bottom of the chambers. Synthetic air or dinitrogen was supplied via the gas inlet to aerate the slurries from below. Gas was released from the gas outlet.

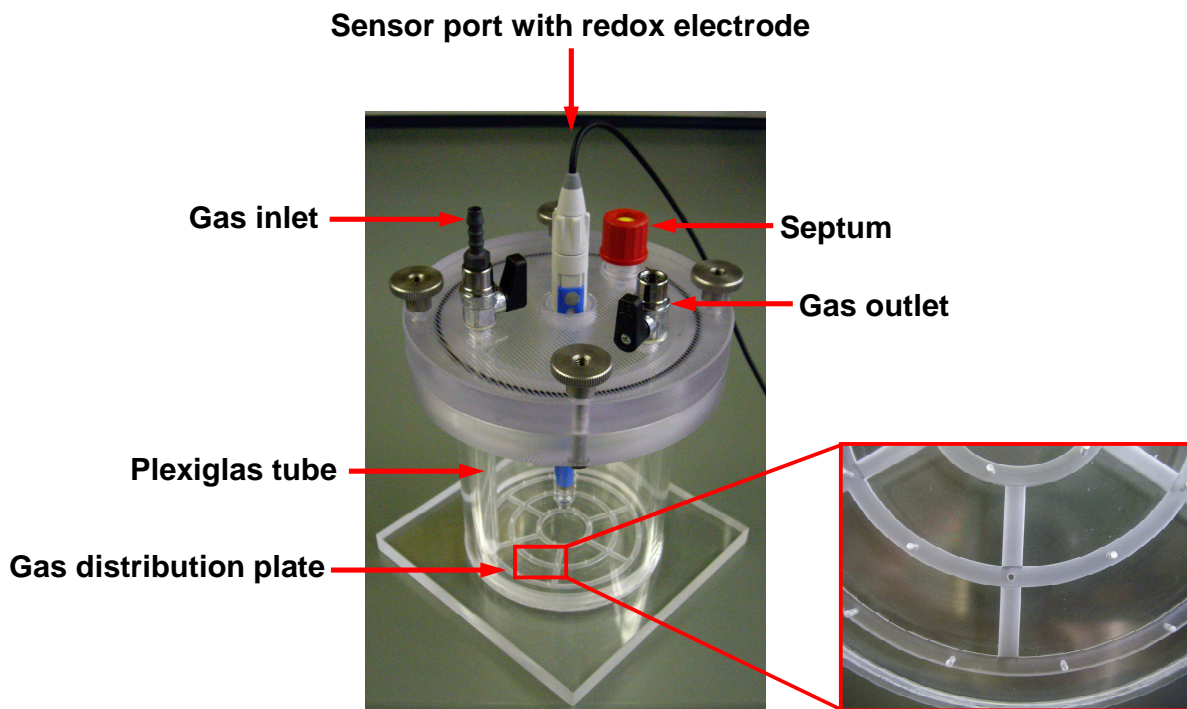


Figure 5. Incubation chamber. Sensor port was used for pH and redox potential measurements. Sterile synthetic air or dinitrogen were supplied at a gas inlet, and slurries were aerated from below by a gas distribution plate. Liquid samples were taken at the septum with a sterile syringe. Gas was released from the gas outlet. Valves of gas inlet and outlet were closed during anoxic incubation, but at the oxic phases synthetic air was permanently flushed through the soil slurry.

2.3.2.2. Incubation Conditions

50 g of field fresh, sieved soil was filled in a chamber, 250 ml sterile oxic or anoxic ddH₂O was added, chambers were closed, and the slurries were mixed thoroughly by manual shaking. The chambers were divided into four groups: anoxic controls (permanently anoxic, no substrate supplementation), redox controls (changing O₂ status, no substrate supplementation), cellobiose-supplemented treatments (changing O₂ status), and carboxymethyl-cellulose (CMC)-supplemented treatments (changing O₂ status). Chambers were then flushed with sterile dinitrogen (100% Rießner, Germany, 3 replicates) or with synthetic air (80% dinitrogen, 20% O₂, Rießner, Germany; 9 replicates) for 1 hour. Cellobiose and CMC were periodically supplemented (0.5 mM substrate per pulse), i.e., the substrate was supplemented after 48, 96, and 168 hours of incubation. Total amounts of cellobiose and CMC pulsed were 0.6 mmol, which corresponds to 7.2 mmol and 4.8 mmol of carbon, respectively. After 48 hours the chambers were flushed with dinitrogen for 1.5 hours to achieve anoxic conditions in treatments formerly incubated under O₂ containing atmosphere. Re-aeration with O₂ was performed after 168 hours in redox controls and substrate-supplemented treatments by constant flushing with synthetic air until the end of incubation. Duration of the anoxic incubation was used since similar periods of O₂ depletion are likely to occur in upland soils (Pett-Ridge and Firestone 2005). Treatments were set up in triplicates and chambers were incubated at 20°C. 2

ml of the liquid phase were taken for determination of soluble organic compounds (2.4.7). Additionally 2 ml liquid sample were taken after 0, 48, 102, 168, and 196 hours for molecular analyses (2.5), i.e., quantification of the transcript numbers of the following domain- and family-level taxa (2.5.10): *Archaea*_{Total}, *Bacteria*_{Total}, *Micrococcaceae/Cellulomonadaceae* (phylum *Actinobacteria*), *Kineosporiaceae/Nocardoidaceae* (phylum *Actinobacteria*), Cluster I *Clostridiaceae* (phylum *Firmicutes*), *Planctomycetaceae* (phylum *Planctomycetes*), the new taxa 'Cellu1-3' (phylum *Bacteroidetes*), 'Sphingo1-4' (phylum *Bacteroidetes*), and 'Deha1' (phylum *Chloroflexi*).

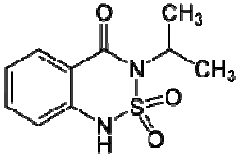
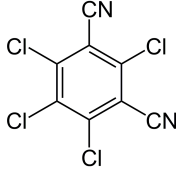
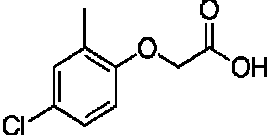
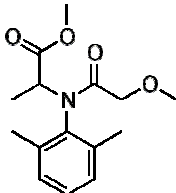
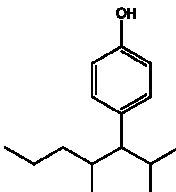
2.3.3. Treatments to Resolve Impact of Pesticides on the Degradation of Saccharides

Oxic and anoxic soil treatments were set up to analyse the impact of pesticides on the aerobic and anaerobic degradation of cellulose and cellobiose, and the involved prokaryotic soil community. Prokaryotic taxa that were previously identified to be part of the active cellulolytic and saccharolytic soil community (2.5.6) were quantified by 16S rRNA gene based quantitative PCR (qPCR; 2.5.10). Five pesticides were tested for potential inhibitory effects in short-term preincubations (2.3.3.1). The lack of an aqueous phase with defined volume in cellulose-supplemented soil treatments prohibits expression of values in mM. Therefore, in all treatments determining pesticide impact any concentration is given in $\mu\text{mol g}_{\text{soil DW}}^{-1}$.

2.3.3.1. Pesticides

Five xenobiotic compounds (Table 5) were tested for their potential to impact on the degradation of the model substrate cellobiose. Compounds comprise ingredients of commercially available pesticide formulations. The term 'pesticide' is used to denote these compounds throughout the work.

Table 5. Pesticides that were tested.

Active ingredient (IUPAC) ^a	Chemical structure ^b	Ingredient of	DT ₅₀ in soil [d] ^c	
			oxic	anoxic
Bentazon (3-Isopropyl-1-H-2,1,3-benzothiadiazin-4-[3-H]-one-2,2-dioxide)		Herbicides	8 - 102	> 89
Chlorothalonil (2,4,5,6-Tetrachloro-isophthalonitrile)		Fungicides	16	9
MCPA (4-Chloro-2-methylphenoxy acetic acid)		Herbicides	7 - 41	No degradation
Metalaxyl (Methyl N-[methoxyacetyl]-N-[2,6-xyllyl]-DL-alaninate)		Fungicides	36	66
Nonylphenol (4-(2,4-Dimethylheptan-3-yl)-Phenol)		Fungicides and Herbicides	30	300

^a Pesticide name, IUPAC: database of the European commission (http://ec.europa.eu/sanco_pesticides/public/index.cfm, last update: 11.10.2010) and of the United States Environmental Protection Agency, (EPA; <http://www.epa.gov/pesticides/index.htm>, last update: 26.01.2011).

^b Chemical structure without copyright available at www.wikipedia.org.

^c DT₅₀, Half-life time in days at 20°C. Values were taken from mentioned databases.

Concentration of pesticides in preincubations exceeded the concentrations usually applied in agriculture (=‘high’, Table 6). Pesticides that had negative effects on the degradation of cellobiose were chosen for further analyses (3.5., 3.5.1.). The impact of recommended concentration of pesticides (=‘low’, Table 6) was also analysed in these subsequent experiments (2.3.3.2).

Table 6. Concentration of pesticides in treatments.

	Amount of pesticide [mg replicate ⁻¹]		Concentration of pesticides [$\mu\text{mol g}_{\text{soil DW}}^{-1}$] ^a		Reference
	'low'	'high'	'low'	'high'	
Cellulose-supplemented					
Bentazon	0.5	53.8	0.1 (1x)	8.5 (112x)	Beulke et al. 2005
Chlorothalonil	-	48.0	-	6.8 (10x)	Katayama et al. 1991
MCPA	0.1	16.0	0.01 (1x)	2.4 (143x)	McGhee and Burns 1995
Metalaxyl	-	3.2	-	0.4 (33x)	Monkiedje et al. 2007
Nonylphenol	2.5	20.5	0.4 (1x)	3.5 (8x)	Okai et al. 2000b
Cellulose-supplemented					
Bentazon	-	24.0	-	2.4 (32x)	Galhano et al. 2009
MCPA	-	20.1	-	2.4 (143x)	McGhee and Burns 1995
Nonylphenol	-	22.0	-	2.4 (6x)	Giger et al. 1984

^a In parenthesis: approximated concentration above recommended application dose.

-, Pesticide was not applied in 'low' concentration.

Field fresh, sieved soil was mixed with 2.5 volumes of sterile oxic or anoxic ddH₂O, manually mixed, and placed on ice for flushing with sterile dinitrogen (100%; Rießner, Lichtenfels, Germany) or sterile air for 1 hour. Soil slurries were homogenized on end-over-end shaker for 1.5 hours at 5°C and were then divided into 76 ml aliquots. Aliquots were transferred into butyl rubberstoppered 125 ml (anoxic) or 500 ml (oxic) flasks (Müller & Krempel, Bülach, Switzerland) that contained the corresponding amount of pesticide (Table 6). 4 ml of a cellobiose stock solution (5 mM) were added per replicate what corresponds to a concentration of cellobiose of 0.25 μM or 0.75 $\mu\text{mol g}_{\text{soil DW}}^{-1}$. Treatments without addition of pesticides were used to analyse unaffected cellobiose degradation. All treatments were set up in duplicates. Incubation atmosphere was dinitrogen or air, and incubation was performed on an end-over-end shaker at 15°C for 24 hours (oxic) or 48 hours (anoxic). 2 ml of the liquid phase were taken every 2 – 6 hours for determination of soluble organic compounds (2.4.7). Pesticide half-life times (Table 5) exceeded the incubation time manifold. Therefore, concentration of pesticides was not monitored.

2.3.3.2. Impact of Bentazon, MCPA, and Nonylphenol on the Degradation of Cellobiose

The impact of Bentazon, MCPA, and Nonylphenol on the aerobic and anaerobic degradation was analysed in soil slurries, which were prepared as explained above (2.3.3.1). All treatments were prepared in triplicates and pesticides were added at either a 'low' or a 'high' concentration (Table 6). 2 ml of the liquid and 0.2 ml of the gaseous phase were taken every 2 – 6 hours with sterile syringes for analysis of methane, carbon dioxide and molecular hydrogen (2.4.6), soluble organic compounds (2.4.7), and pH measurements (2.4.4). Concentration of pesticides was not determined.

2.3.3.3. Impact of Bentazon, MCPA, and Nonylphenol on the Degradation of Cellulosic Paper Sheets

Aerobic and anaerobic treatments were prepared to analyse the impact of pesticides on the degradation of crystalline cellulose. 50 g of field fresh, sieved soil was thoroughly mixed with powdered Bentazon, MCPA or Nonylphenol and filled in butyl-rubber-stoppered 125-ml (anoxic treatment) or 500-ml (oxic treatment) flasks. Concentration of pesticides was $2.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$ (Table 6). Soil without pesticides was used as control. One sheet of cellulose paper (Whatman, Maidstone, UK; > 98% α -cellulose) was added to every flask. Therefore, sheets were dried for 4 hours at 40°C, weighed on an analytical balance (Analytic AC 120 S, Sartorius, Garching, Germany), cut into smaller pieces, and inserted into the soil, so that the cellulose pieces were completely covered by soil (Munier-Lamy and Borde 2000; 2.4.8). The flasks were closed and flushed for 30 minutes with sterile dinitrogen or air, respectively. Cellulose-supplemented treatments were prepared in seven replicates ($0.163 \pm 0.004 \text{ g cellulose replicate}^{-1}$). A control for measuring microbial processes related to soil indigenous carbon turnover was analyzed in triplicate (2.3). Incubations were performed at 15°C for 70 days.

Every 14 days, gas samples were taken for analysis of methane, carbon dioxide and molecular hydrogen (2.4.6), and afterwards headspace was exchanged with dinitrogen or air. One replicate per treatment was excluded from the running experiment and used for subsequent (destructive) analyses, i.e., measurement of cellulose weight loss (Munier-Lamy and Borde 2000; 2.4.8), concentration of pesticides (2.4.9), soluble organic compounds (2.4.7), inorganic compounds (2.4.10), and pH measurements (2.4.4). Remaining soil of each replicate was stored at -80°C for 16S rRNA gene-based analyses of cellulolytic and saccharolytic taxa (2.3.1) by quantitative PCR (qPCR; 2.5.10), i.e., quantification of the transcript numbers of the following domain- and family-level taxa (2.5.10): *Bacteria*_{Total}, *Micrococcaceae*/ *Cellulomonadaceae* (phylum *Actinobacteria*), Cluster I *Clostridiaceae* (phylum *Firmicutes*), *Planctomycetaceae* (phylum *Planctomycetes*), and the new taxa 'Cellu1-3' and 'Sphingo1-4' (phylum *Bacteroidetes*). QPCR was performed with RNA derived from the beginning and at the end of incubation (after 70 days) in Bentazon- and MCPA-supplemented treatments.

2.3.3.4. Quantification of Pesticide Effects on Processes Linked to the Degradation of Cellulose and Cellobiose

The amount of degraded saccharides, produced carbon dioxide, molecular hydrogen, and ferrous iron (Fe^{2+}) in pesticide-supplemented compared to pesticide-free control treatments was used to estimate quantitative effects of pesticides on degradation processes. Changes in concentrations between starting time point and 70 days (end of experiment) were calculated in experiments with cellulose supplementation (2.3.3.3). Cellulose degradation was determined by the loss in dry weight of supplemented paper sheets and values were converted to concentrations in $\mu\text{mol g}_{\text{soil DW}}^{-1}$ assuming that 1 mol of cellulose is identical to 1 mol glucose equivalents ($\text{MW} = 180 \text{ g mol}^{-1}$), independent of polymer length. Changes in concentrations in cellobiose-supplemented treatments (2.3.3.2) were calculated between starting time point and 10 hours (oxic) and 42 hours (anoxic), i.e., time point at which complete consumption of cellobiose and glucose in pesticide-free treatments occurred. Since no glucose molecules were supplied, consumption of glucose was calculated based on the amount of glucose-equivalents supplied in the form of cellobiose. Relative inhibition of degradation processes was calculated based on these values, and is given in percent.

2.4. Analytical Methods

2.4.1. Dry Weight and Gravimetric Water Content of Soil

The dry weight of soil (DW) was determined by weighing field fresh, sieved soil before and after drying for 24 hours at 105°C (Blume et al. 2010) in a drying cabinet (Thermo Fisher Scientific, Waltham, USA). Gravimetric water content was calculated according to Equation 1.

Equation 1. Gravimetric water content.

$$W_g = \frac{m_{FW} - m_{DW}}{m_{DW}} \cdot 100\%$$

W_g , gravimetric water content; m_{FW} , fresh weight of soil; m_{DW} , dry weight of soil (Blume et al. 2010)

2.4.2. Total Carbon and Nitrogen Content

Total carbon (total C) and nitrogen (total N) content was determined by co-workers of the Department for Soil Ecology, University of Bayreuth. Soil was dried for 24 hours at 105°C and powdered in a vibration mill (Type MM2, Retsch, Haan, Germany). Dried samples were analysed in an elemental analyzer (CHN-O-rapid, Foss-Heraeus, Hanau, Germany).

2.4.3. Soil Texture and Soil Type

The ratio of clay, silt, and sand particles defines the soil texture. This ratio was determined by the Department of Soil Ecology, University of Bayreuth (Kretschmar 1996) and soil was classified as a clay loam (Lt₂, German soil classification; Eckelmann 2005). Soil type was a Dystric Cambisol (FAO-classification; Fuka et al. 2008).

2.4.4. pH

pH was measured with a combination pH electrode (U457-S7/110, Ingold, Steinbach, Germany) and a pH-Meter (WTW pH 330, Wissenschaftliche Werkstätten, Weilheim, Germany). pH was determined in field fresh soil of every sampling date (2.2). Soil was sieved and mixed with 2.5 volumes of sterile ddH₂O or CaCl₂, respectively. Suspensions were homogenized by manual shaking for 5 minutes and pH was measured after stabilization for 5 minutes. pH in treatments experiments (2.3.1, 2.3.3, 2.3.2) was measured in 2 ml aliquots directly after sampling.

2.4.5. Redox Potential

The redox potential of treatments in self constructed incubation chambers (2.3.2) was measured by an EMC 30 L electrode (Sensortechnik Meinsberg, Ziegra-Knobelsdorf, Germany) and a pH-Meter (WTW pH 330, Wissenschaftliche Werkstätten, Weilheim, Germany). Incubation chambers (Figure 5) were shaken briefly, the redox electrode was inserted through the sensor port into the slurry, and the redox potential was measured after it stabilized for 1 minute.

2.4.6. Gases

Concentrations of carbon dioxide, methane, and molecular hydrogen were determined with Hewlett Packard 5890 Series II gas chromatographs (GC) (Palo Alto, USA). All columns were obtained from Alltech (Alltech, Unterhaching, Germany). GC parameters are listed in Table 7. Overpressure in the incubation flasks was measured directly before sampling with a needle gauge (DGM 2120, DGM, Horgen, Switzerland). Gas samples were taken with sterile syringes that were flushed with sterile argon before and after the measurements. Injection volume was 200 µl.

Table 7. Parameters of GC measurements.

	CO₂	CH₄	H₂
Detectors	TCD	FID	TCD
Column	Chromosorb 102, 2m x 1/8"	Molecular Sieve, 2 m x 1/2"	Molecular Sieve, 2 m x 1/8"
Oven temp. [°C]	40	60	50
Injection temp. [°C]	150	120	150
Detector temp. [°C]	175	150	175
Carrier gas	Helium	Helium	Argon
Flow rate [ml min ⁻¹]	15	40	33
Retention time [min]	1.9	1.7	0.6
Reference	Daniel et al. 1990	Küsel and Drake 1995	Daniel et al. 1990

temp., Temperature.

FID, Flame ionisation detector.

TCD, Thermal conductivity detector.

The area of the gas peaks was integrated with the Software EuroChrom (Version V3.05, Knauer, Berlin, Germany) and the use of external standards with known gas concentrations. Gas concentrations in the flasks were calculated, including overpressure in incubation flasks, actual room temperature, actual air pressure, and volume of gas and liquid phase (Equation 2 - Equation 6).

The total amount of a single gas (n_{ges}) in the incubation flasks is comprised of its proportion in the gaseous (n_{gas}) and in the liquid phase, i.e., the physically dissolved amount ($n_{gel,p}$) (Equation 2). For carbon dioxide the chemically dissolved amount ($n_{gel,c}$, carbon dioxide in the form of HCO_3^-) has also to be allocated (Equation 6).

Equation 2. Total amount of gas.

$$n_{ges} = n_{gas} + n_{gel,p} + n_{gel,c}$$

The amount of gas in the gaseous phase (n_{gas}) was calculated with Equation 3.

Equation 3. Amount of gas in the gaseous phase.

$$n_{gas} = V_{gas} \cdot \frac{c}{V_{akt,mol}} \cdot \frac{p_{akt} + p_{\ddot{u}}}{p_{akt}}$$

n_{gas} , amount of gas in gaseous phase (μmol); V_{gas} , volume of gas phase (ml); c , measured gas concentration (ppm); $V_{\text{akt,mol}}$, molar volume of gas under actual conditions (ml); p_{akt} , actual air pressure (mbar); $p_{\text{ü}}$, overpressure in incubation flasks (mbar).

The molar gas volume at actual conditions ($V_{\text{akt,mol}}$) was determined based on the ideal gas law (Equation 4).

Equation 4. Ideal gas law.

$$V_{\text{akt,mol}} = \frac{p_1 \cdot V_1 \cdot T_{\text{akt}}}{T_1 \cdot p_{\text{akt}}}$$

p_1 , standardized air pressure (mbar); V_1 , standardized molar gas volume (ml); T_1 , standardized temperature (K); T_{akt} , actual temperature (K).

The amount of gas physically and chemically dissolved in the liquid phase is based on Equation 5 and Equation 6. Bunsen solubility coefficient (α) is variable and is dependent on the gas type and the actual temperature (Blachnik 1998; Table 8).

Equation 5. Amount of physically dissolved gas.

$$n_{\text{gel,p}} = V_{\text{fl}} \cdot \frac{c}{V_{\text{akt,mol}}} \cdot \alpha \cdot \frac{p_{\text{akt}} + p_{\text{ü}}}{p_{\text{akt}}}$$

V_{fl} , volume of liquid phase (ml);

Equation 6. Amount of chemically dissolved gas.

$$n_{\text{gel,c}} = n_{\text{gel,p}} \cdot 10^{-pK_a + pH}$$

Table 8. Bunsen solubility coefficients of carbon dioxide, methane, and molecular hydrogen at different temperatures (Blachnik 1998).

	Bunsen solubility coefficient α (in water)	
	293.15 K (20°C)	298.15 K (25°C)
CO₂	0.850	0.740
CH₄	0.032	0.029
H₂	0.018	0.015

2.4.7. Soluble Organic Compounds

Concentration of alcohols, fatty acids, and sugars was measured by high performance liquid chromatography (HPLC; Ehrlich et al. 1981; Matthies et al. 1993) in liquid samples of microcosm experiments (2.3). Soluble organic compounds in cellulose-supplemented treatments (2.3.3.3) were determined after mixing soil with 2.5 volumes of ddH₂O and homogenization on an end-over-end shaker for 10 minutes at 5°C. Liquid samples were centrifuged (1- 15K Sartorius microcentrifuge, Sigma Laborzentrifugen, Osterode am Harz, Germany; 13,000 x g, 10 minutes, 4°C) and supernatant was microfiltrated (HPLC nylon filter, pore volume 0.2 µm, Infochroma, Zug, Switzerland) into flange bottles with aluminium caps (VWR International, Darmstadt, Germany). Samples were directly analysed after filtration in a Hewlett Packard 1090 Series II HPLC system (Palo Alto, USA) equipped with an autosampler (Table 9). Injection volume was 20 µl. External standards with known concentrations of soluble organic compounds were used for quantification.

Table 9. Parameters of HPLC system for soluble organic compounds.

Hewlett Packard 1090 Series II	
Detectable compounds	Alcohols, fatty acids, sugars
Precolumn	Carbo-H 4 x 3 mm ID
Column	Aminex Ion Exclusion HPX-87H; 300 x 7.8 mm (BioRad, Richmond, USA)
Detectors	G1362A RID, G1314B VWD (210 nm) (Series 1200, Agilent Technologies, Böblingen, Germany)
Mobile phase	H ₃ PO ₄ (4 mM)
Oven temp. [°C]	60
Flow rate [ml min ⁻¹]	0.8
Software	ChemStation (Agilent Technologies, Böblingen, Germany)

temp., Temperature.

RID, Refractive index detector.

VWD, Variable wavelength detector.

2.4.8. Cellulose

Degradation of cellulose (2.3.3.3) was determined by measuring cellulose weight loss of buried cellulosic paper sheets (Munier-Lamy and Borde 2000). This procedure is a suitable method to measure degradation of crystalline cellulose (Hiroki and Watanabe 1996; Jacobson and Jacobson 1998; Kurka 2001; Munier-Lamy and Borde 2000). Sheets were dried at 40°C for 4 hours in a drying cabinet (Thermo Fisher Scientific, Waltham, USA) and weighed on a MC1 analytic AC 120 S balance (AS Wägetechnik, Garding, Germany) before insertion into the soil. Sheets were gently removed with forceps after incubation. Sheets were washed with sterile ddH₂O to remove attached soil particles, dried, and weighed. The difference in cellulose weight loss was used for calculation of the potential inhibition by pesticides (2.3.3.4).

2.4.9. Pesticides

Pesticides were extracted and quantified in pesticide-supplemented treatments (2.3.3.3; Liu et al. 2011). The pesticide extraction procedure was optimized and the extraction efficiencies were $102.4 \pm 4.1\%$ for Bentazon, $98.3 \pm 2.8\%$ for MCPA, and $92.1 \pm 2.5\%$ for Nonylphenol. Bentazon and Nonylphenol were extracted with 70% ethanol, and MCPA with 10 mM NaOH (in triplicates). One volume of soil was mixed with three volumes of solvent. Mixtures were incubated for 2 hours at 15°C on an end-over-end shaker and centrifuged (13,000 rpm, 10 minutes, room temperature). Samples were microfiltered and injected in a Agilent Technologies 1200 Series HPLC instrument (Agilent Technologies, Böblingen, Germany) that was equipped with a 1200 Series diode-array detector (Agilent 1200 series, Agilent Technologies, Böblingen, Germany) (Table 10). Pesticides were separated with on a MultoHigh 100 RP 18-5 μ column (250 x 4 mm) with a pre-column (20 x 4 mm; CS Chromatographie, Langerwehe, Germany) at constant temperature (30°C). Peak purity was confirmed by online spectra from 220 to 340 nm. External standards with known concentrations of pesticide were used for calibration. Signals were recorded at 230 nm with the software ChemStation (Version B.04.01; Agilent Technologies, Böblingen, Germany)

Table 10. Parameters of pesticide measurements by HPLC.

	Bentazon	Nonylphenol	MCPA
Injection temp. [°C]	30	30	30
Detector temp. [°C]	30	30	30
Mobile phase	50:50 (v/v %) Acetonitrile : C ₂ H ₃ NaO ₂ (20 mM)	70:30 (v/v %) Acetonitrile : ddH ₂ O	50:50 (v/v %) Acetonitrile : C ₂ H ₃ NaO ₂ (20 mM)
Flow rate [ml min ⁻¹]	1.0	2.5	1.0
Retention time [min]	7 - 9	11 - 12	7 - 9
Reference	This work	This work	Liu et al. 2011

temp., Temperature.

2.4.10. Inorganic Compounds

2.4.10.1. Nitrate (NO_3^-) and Total Amounts of Ammonium (NH_4^+), Iron, Manganese, and Sulphate

Compounds were analysed by standardized colorimetric methods by co-workers of the Institute of Central Analytics, University of Bayreuth. External standards with known concentrations were used for quantification.

2.4.10.2. Ferrous Iron (Fe^{2+})

Concentrations of ferrous iron were determined photometrically (Tamura et al. 1974). 20 μl sample were mixed with 980 μl HCl (0.5 N), incubated for 1 hour at room temperature, and microfiltrated to remove soil particles. 450 μl sample were mixed with 50 μl acetate buffer (200 g $\text{CH}_3\text{COONH}_4$, 250 ml CH_3COOH , ad 500 ml ddH_2O) and 50 μl of a phenanthroline solution (1 g 1,10-Phenanthroline-chloride-monohydrate ad 200 ml ddH_2O), leading to the development of a red-coloured Fe-phenanthroline-complex. 160 μl of the mixture were immediately transferred to microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) and extinction was measured at 512 nm by a μQuant microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany). The software Gen5 (Version 1.04.5; BioTek, Bad Friedrichshall, Germany) and external standards were used for quantification.

2.5. Molecular Methods

2.5.1. Extraction of Nucleic Acids

Nucleic acids, i.e., DNA and RNA, were co-extracted according to a modified protocol of Griffiths et al. 2000. All steps were performed in certified RNase- and DNase-free tubes. Filtered tips were used to avoid contamination of nucleic acids with hydrolytic enzymes. Centrifugation was carried out on an analytical centrifuge (1-15K Sartorius microcentrifuge, Sigma, Osterode am Harz, Germany) at 13,000 $\times g$ for 5 minutes at 4°C unless otherwise stated. 2 ml soil slurry were transferred to 2 ml screw-capped tubes (VWR International, Darmstadt, Germany), centrifuged, and supernatant was discarded. 1 g of baked (12h, 200°C) zirconium beads (0.5 g \varnothing 0.1 mm, 0.5 g \varnothing 0.5 mm; CarlRoth, Karlsruhe, Germany), 500 μl preheated extraction buffer (60°C, 5% CTAB, 350 mM NaCl, 120 mM potassium phosphate buffer [containing KH_2PO_4 and K_2HPO_4], pH 8), and 500 μl phenol/chloroform/isoamylalcohol (25:24:1, pH 8) were added. Samples were bead beaten two times at 5.5. m/s in a Bead Beater (FastPrep FP 120, Thermo Savant, USA) for 30 seconds and centrifuged. Supernatant was transferred to sterile 1.5 ml tubes, 500 μl chloroform/isoamylalcohol (24:1) were added, samples were mixed, and centrifuged. Supernatant was transferred to sterile 2 ml tubes and 2 volumes of precipitation buffer (30% PEG6000, 0.1 M HEPES, pH 7) were added until the solution was clear. Nucleic acids were precipitated at room temperature (20 – 25°C) for 2 hours and centrifuged to pellet nucleic acids. The pellet was washed with 500 μl ice cold ethanol (70%, -20°C) to dissolve co-precipitated salts, centrifuged, and the supernatant was discarded. Nucleic acids were dried at room temperature and dissolved in 52 μl DNase/RNase-free ddH_2O . Nucleic acid extraction was checked spectrophotometrically (2.5.3.1) and extracts were stored at -80°C.

2.5.2. Separation of DNA and RNA

2.5.2.1. Solid Phase Columns

Co-extracted DNA and RNA (2.5.1) were separated with the QIAGEN RNA/DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The procedure was started at step 3 of the protocol for 'Isolation of total RNA and genomic DNA from Bacteria', a protocol suitable for total RNA amounts smaller than 40 µg. After separation DNA was dissolved in 50 µl, RNA in 30 µl DNase/RNase-free ddH₂O. DNA and RNA were quantified (2.5.4.2) and samples were stored at -80°C.

2.5.2.2. Enzymatic Separation

An alternative enzyme-based method was applied to separate nucleic acids. RNA was obtained by digestion of DNA with DNase I (1U µl⁻¹; Fermentas, St. Leon-Roth, Germany). 3 µl reaction buffer (10x) and 1 µl DNase I were mixed with 30 µl co-extract and samples were incubated on a thermomix comfort (Eppendorf, Hamburg, Germany) at 37°C for 45 minutes under constant shaking (300 rpm). DNA was obtained by digestion of RNA with RNase A (10 µg µl⁻¹; Fermentas, St. Leon-Roth, Germany). 1 µl RNase A were added to 20 µl co-extract and samples were incubated for 20 minutes at room temperature (20 – 25°C). Enzymatic digestions were stopped by purification of nucleic acids by isopropanol/Na-acetate precipitation (2.5.3.1).

2.5.3. Purification and Precipitation of Nucleic Acids

2.5.3.1. Isopropanol/Sodium Chloride Precipitation

Nucleic acids that were subjected to DNase I or RNase A digestion (2.5.2.2) were purified by isopropanol precipitation (Sambrook and Russell 2001). 0.7 volumes of ice cold isopropanol (100%, -20°C) and 0.1 volume of NaCl (5 mM) were added to 1 volume of nucleic acids. Samples were incubated for at least 12 hours at -20°C, and nucleic acids were precipitated by centrifugation (18,000 x g, 45 minutes, 15°C). The DNA or RNA pellet was washed with 500 µl ice cold ethanol (70%, -20°C), dried at room temperature, and dissolved in 22 µl DNase/RNase-free ddH₂O. 2 µl of nucleic acid solution were used for quantification of DNA or RNA by fluorescence based methods (2.5.4.2). DNA and RNA samples were stored at -80°C.

2.5.3.2. Gel Extraction

PCR products amplified for tRFLP analyses (2.5.11) and PCR products for the construction of gene libraries (2.5.12) were purified by gel extraction (Montage Gel Extraction Kit, Millipore, Bedford, USA) after the manufacturer's protocol. Samples were loaded on a 1% agarose gel that was prepared with modified TAE buffer (40 mM Tris-Acetate, 0,1 mM Na₂EDTA, pH 8,0; Millipore, Bedford, USA). Agarose gel electrophoresis was performed for 1 hour at 70 mV as described (2.5.5). Bands were excised under UV-light with a sterile knife and loaded on Montage DNA Gel Extraction Device.

2.5.3.3. Filter Plates

PCR products that were subjected to mung bean endonuclease digestion (2.5.11.1) and PCR products representing clone insert sequences or re-amplified 16S rRNA genes thereof (2.5.9.3, 2.5.10.2) were purified with Millipore PCR₉₆ Cleanup Plates (Millipore Cooperation, Bedford, USA) after manufacturer's protocol. Samples were loaded onto the filters and plates were evacuated for 12 minutes with a suction pump (KNF Neuberger, Balterswil, Switzerland). PCR products were dissolved in 25 μ l tris buffer (10 mM, pH 8.5) and transferred into sterile 0.5 ml tubes (LoBind tubes, Eppendorf, Hamburg, Germany) by pipetting.

2.5.4. Quality Control and Quantification of Nucleic Acids

2.5.4.1. Spectrophotometry

Quality of nucleic acid extraction was determined with a Nanodrop ND-1000 spectrophotometer (PEQLAB Biotechnology GmbH, Erlangen, Germany) and supplied software. Absorption at 260 nm, 280 nm, and 230 nm was measured in 2 μ l sample. One absorption unit at 260 nm (A_{260}) corresponds to a concentration of 50 μ g_{DNA} ml⁻¹ and 33 μ g_{RNA} ml⁻¹, respectively (Lottspeich and Engels 2006; Sambrook and Russell 2001). The attenuation coefficient A_{260}/A_{280} gives information about possible contaminations of nucleic acids with phenol, proteins, or humic acids. An ideal coefficient lies between 1.6 and 2.0 (Sambrook and Russell 2001). Additionally the absorption spectrum should be low at 230 nm, because humic acids exhibit a strong absorption at this wavelength (Tsutsuki and Kuwatsuka 1979). All DNA and RNA samples of the current study showed a good quality and were not contaminated with humic acids.

2.5.4.2. Pico-/RiboGreen-Based Quantification

DNA and RNA samples of nucleic acids after extraction (2.5.2), purified PCR products (2.5.3), and PCR products that were subjected to restriction digestion for tRFLP analysis (2.5.11.2) were quantified with a fluorescence-based method (Sambrook and Russell 2001). Fluorescence-based methods are insensitive to interferences caused by proteins or other contaminations, and allow exact quantification of very low amounts of nucleic acids (minimum: 25 pg_{DNA} ml⁻¹ [Jones et al. 1998] or 20 ng_{RNA} ml⁻¹ [Labarca and Paigen 1980]). Quantification of DNA and RNA was performed in microtiter plates with the Quant-iT-PicoGreen dsDNA reagent Kit and the Quant-iT-RiboGreen Quantitation Reagent Kit (both Invitrogen, Karlsruhe, Germany), respectively. A FLx800 microplate fluorometer (BioTek, Bad Friedrichshall, Germany) was used and quantification was performed according to the manufacturer's protocol. For quantification of RNA an assay suitable for 1 – 50 ng ml⁻¹ was used (i.e., 'low range'). External standards with known concentrations of DNA or RNA were entrained in every measurement. Fluorescence signals were recorded with the software Gen5 (BioTek, Bad Friedrichshall, Germany).

2.5.5. Agarose Gel Electrophoresis

PCR products were qualified with horizontal agarose gel electrophoresis, a method that separates nucleic acids in an electrical field dependent on their size (Aaij and Borst 1972; Sambrook and Russell 2001). Agarose gels (1%) were prepared with 1x TAE buffer (40 mM Tris-HCl, 20 mM CH₃COOH, 1 mM EDTA, pH 8). The buffered mixture was heated in a MWG 756 E microwave (Clatronc, Kempen, Germany) until agarose was fully dissolved. After cool down to 60°C 0.05 mg ml⁻¹ ethidium bromide (3,8-Diamino-5-ethyl-6-phenyl-phenanthridiumbromid) were added for visualization. Ethidium bromide intercalates with DNA and shows a strong fluorescence under UV-light after binding to DNA. Liquid gels were filled in a gel rack, transferred to a migration chamber (Techne, Jahnsdorf, Germany) after solidification, and covered with running buffer (1x TAE). Samples were prepared by mixing 5 µl sample with 1 µl 6 x Blue Orange loading dye (Promega, Madison, USA) or self prepared 6 x loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 55% glycerol). Samples were filled into the gel slots. 2 µl of a DNA ladder (MWM-1, 200 – 10,000 bp, Bilatec, Viernheim, Germany) were used for determination of product length. Electrophoresis was performed at 120 V for 20 – 30 minutes. Visualization of bands by UV-light (302 nm) was carried out on a transilluminator (UVT-20M, Herolab, Wiesloch, Germany) and a photographically based documentation system (Canon PowerShot G5, Canon, Krefeld, Germany; software: Remote Capture).

2.5.6. rRNA Stable Isotope Probing (RNA SIP)

rRNA-based stable isotope probing is a culture-independent method for identification of microorganisms that are responsible for a certain process (e.g., cellulose degradation; Manefield et al. 2002b). In this study, the protocol of Whiteley et al. 2007 was used in a slightly modified form. Stable isotope probing experiments were performed in oxic and anoxic treatments (2.3.1)

[¹³C]-labeled substrates are added to the environmental sample and incubated (Figure 6). Microbial assimilation of [¹³C]-labeled substrates results in the incorporation of heavy carbon isotopes into their biomass, e.g., nucleic acids. As a consequence, the buoyant density of DNA and RNA increases (Meselson and Stahl 1958). Total nucleic acids are extracted and RNA is separated from DNA. [¹³C]-labeled 'heavy' RNA is then separated from the 'light' [¹²C]-RNA by isopycnic ultracentrifugation and subsequent fractionation of the gradients. RNA of every gradient fraction is precipitated and reversely transcribed into complementary DNA (cDNA). CDNA is used for identification of tRFs related to [¹³C]-labeled organisms by molecular methods (e.g., tRFLP fingerprinting [2.5.11], cDNA based gene libraries [2.5.12]). However, it is likely that 'heavy' and 'light' RNA are not fully separated during centrifugation. This may be due to insufficient labeling of 'heavy' RNA or co-migration of unlabeled 'light' RNA with high G+C contents (causing higher buoyant densities) towards the 'heavy' fractions (Lüders et al. 2004; Manefield et al. 2002a; Rangel-Castro et al. 2005). Combination of different molecular tools (e.g., tRFLP and cloning) should be taken into account to avoid false interpretation of results only gained by one methodological approach (Bent and Forney 2008).

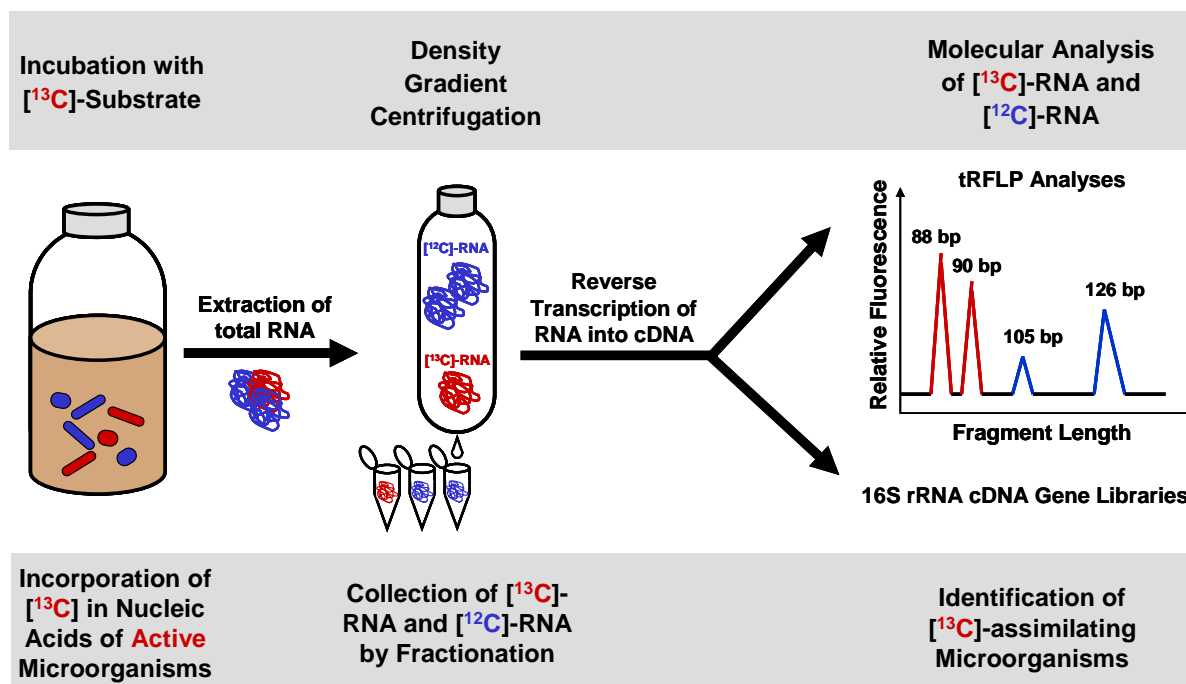


Figure 6. Schematic protocol of stable isotope probing (SIP). See text for details.

2.5.6.1. Density Gradient Centrifugation

A gradient solution (buoyant density: $1.790 \pm 0.005 \text{ g ml}^{-1}$) was prepared that contained 4.61 ml caesium trifluoroacetate (CsTFA, buoyant density: $2.0 \pm 0.05 \text{ g ml}^{-1}$, GE Healthcare, Buckinghamshire, UK), 0.175 ml formamide and a variable amount of gradient buffer (100mM KCl, 100 mM Tris, 1 mM EDTA, pH 8). 0.8 – 1.2 ml of gradient buffer were added until the gradient solution had a buoyant density of $1.790 \pm 0.005 \text{ g ml}^{-1}$. Density of the gradient solution was determined at 25°C. 500 ng of RNA were mixed with 1 ml of gradient solution and transferred into a 4.9-ml-centrifugation tube (OptiSeal Polyallomer Tube, 13 x 48 mm; Beckmann, Palo Alto, USA). The tubes were filled with gradient solution, balanced until a maximal difference of 0.05 g was achieved and closed with a plug. Tubes were placed in an ultra-vertical VTi 65.2 Rotor Package supplied with aluminium spacers (Beckham Coulter, Fullerton, USA). Screws were closed with a torque wrench adapter (200 inch-pounds, Beckham Coulter, Fullerton, USA) to a bolting torque of 60 inch-pounds. $[^{12}\text{C}]$ - and $[^{13}\text{C}]$ -RNA were separated by isopycnic centrifugation at $130,000 \times g$ (37,800 rpm) at 20°C for 67 hours in a LE-70 ultra centrifuge (Beckman Coulter, Fullerton, USA). The rotor ran out non-braked. All gradients were set up with the same gradient solution to minimize potential differences that might otherwise occur by the use of gradient solutions with similar but non identical densities. Isopycnic gradients prepared from soil treatments (2.3.1) containing the same saccharide (including both the oxic and anoxic treatments) were centrifuged simultaneously. In total, 45 gradients (15 for cellulose-, 15 for cellobiose-, and 15 for glucose-supplemented treatments) were processed in three centrifugation runs. A tube containing gradient solution without RNA (= Blank) was entrained in every run to measure the density of the fractions of the gradients (2.5.6.3).

2.5.6.2. Fractionation of Gradients

Fractionation of the gradients was performed manually by a suction pump (Econo Pump1, BioRad, Hercules, USA) (Manefield et al. 2002a). The centrifugation tubes were vertically fixed in a rag. A silicon hose (inner diameter: 1.6 mm) that was linked to the suction pump was equipped with a sterile needle (23G x 1") and inserted carefully into the tube underneath the plug. A second needle was used to drill a hole into the bottom of the tubes. Out flowing gradient solution was collected after the suction pump was started (flow rate: 0.45 ml min^{-1}) and sterile water (coloured with brilliant blue) was pumped onto the gradient solution (Figure 7). Eleven fractions (~400 μl each) were collected in 60-second-intervals from every gradient. The eleventh fraction was contaminated with coloured water and therefore discarded from the analyses.

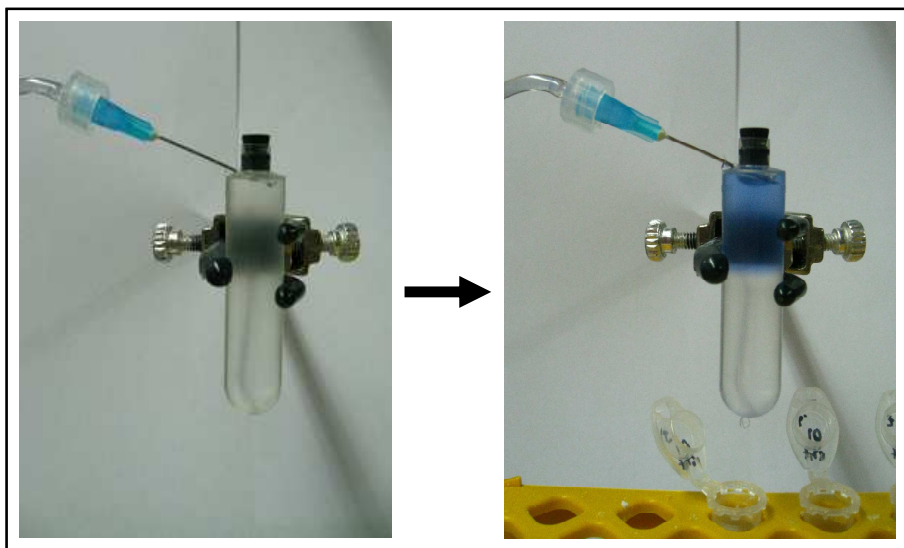


Figure 7. Fractionation of gradients. See text for details.

2.5.6.3. Measurement of the Density of the Fractions

The buoyant density of fractions 1 – 10 was determined in the blank gradient (i.e., without RNA) of each centrifugation run (Manefield et al. 2002b). The fractions were tempered to 25°C in a water bath and extensively mixed. The weight of the tubes was measured on an analytical balance (Analytic AC 120 S, Sartorius, Garching, Germany) before and after 100 μl of the fraction were removed. The density of every fraction was determined 10 times. Two fractions that presumably represented labeled 'heavy' RNA (corresponding to fraction numbers three and four, buoyant density $1.813 \pm 0.001 - 1.821 \pm 0.005 \text{ g ml}^{-1}$) were pooled. Furthermore, two fractions that represented unlabeled 'light' RNA (corresponding to fraction numbers eight and nine, buoyant density $1.767 \pm 0.000 - 1.776 \pm 0.000 \text{ g ml}^{-1}$) (Manefield et al. 2002b) were pooled. Pooled fractions were used for further analyses.

2.5.6.4. RNA Precipitation

RNA in 'light' and 'heavy' fractions was precipitated according to Degelmann et al. 2009a. 200 μl of each fraction were mixed with 130 μl NaCl (3 M, pH 5.2), 13.6 μl glycogen (10 mg ml^{-1}), and 1,000 μl ice cold ethanol (96%). Samples were incubated for at least 12 hours at -20°C . RNA was precipitated by centrifugation (13,000 $\times g$, 20 minutes, 4°C). The supernatant was discarded, the RNA pellet washed with 500 μl ice cold ethanol (70% in RNase-free ddH_2O , -20°C), and dried at room temperature. The RNA was dissolved in 22 μl RNase-free ddH_2O and 2 μl of RNA solution were used for quantification by fluorescence based methods (2.5.4.2). RNA samples were directly used for reverse transcription of RNA into cDNA (2.5.7).

2.5.7. Reverse Transcription of RNA into cDNA

Reverse transcriptase of RNA into single-stranded complementary DNA (cDNA) was performed with the SuperScript VILO cDNA Synthesis Kit or the SuperScript III First-Synthesis System (both: Invitrogen, Darmstadt, Germany). Random hexamers (50 ng μl^{-1}) were used in all reactions. Reverse transcription PCR (RT-PCR) was conducted on a TGradient Thermocycler (Biometra, Göttingen, Germany). Samples were prepared according to the manufacturer's protocols. Annealing of random hexamers to RNA was performed for 10 minutes at 25°C , followed by 90 minutes at $42 - 50^{\circ}\text{C}$ for cDNA synthesis. Reactions were terminated by heat-inactivation of the enzyme for 5 minutes at 85°C . A negative control (RNase-free ddH_2O) and a positive control (control RNA supplied by the manufacturer) were entrained in every reverse transcription. cDNA was stored at -80°C .

2.5.8. *In Vitro* Transcription of DNA into RNA

Single-stranded RNA was prepared by *in vitro* transcription of pGEM-T vector DNA with the T7 transcription kit (Fermentas, St.Leon-Rot, Germany). Single-stranded RNA was used for correction of transcript numbers measured in qPCR assays (2.5.10). Vector DNA was amplified by M13-PCR (2.5.9.3). The PCR product was purified with Millipore PCR₉₆ Cleanup Plates (Millipore Cooperation, Bedford, USA) (2.5.3.3) and quantified with the Quant-iT-PicoGreen dsDNA reagent Kit (2.5.4.2). A maximum of 1 μg of DNA was used for *in vitro* transcription according to the manufacturer's description. A control DNA included in the kit was entrained in the analysis. *In vitro* transcription was performed by 2 hours of incubation at 37°C and reaction was stopped by freezing samples at -20°C . Successful transcription was checked by agarose gel electrophoresis (2.5.5). Template DNA was removed by DNase I digestion (2.5.2.2). Remaining RNA was quantified with the RiboGreen Quantitation Reagent Kit (2.5.4.2).

2.5.9. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method to multiply fragments of DNA or cDNA by several orders of magnitude, i.e., it generates multiple copies of a particular gene sequence. The process of enzymatic amplification of these gene fragments can be divided into three steps: denaturation of the DNA template, annealing of gene-specific primers to single stranded DNA, and elongation of the complementary strand by a thermally stable DNA-polymerase (Saiki et al. 1985; Saiki et al. 1988).

2.5.9.1. Primers and Thermal Protocols

Polymerase chain reactions were performed in a TGradient Thermocycler (Biometra, Göttingen, Germany), a Primus 96 advanced (PEQLAB Biotechnology, Erlangen, Germany), a PeqStar (PEQLAB Biotechnology, Erlangen, Germany), and a LabCycler (SensoQuest GmbH, Göttingen, Germany). Primers for amplification of 16S rRNA genes of *Bacteria* and *Archaea*, and of clone insert sequences (M13-PCR) were purchased from Biomers GmbH (Biomers.net, Ulm, Germany) (Table 11).

Table 11. Primers for amplification of 16S rRNA genes of *Bacteria* and *Archaea*, and of sequences inserted into the pGEM-T vector of clones.

Name of Primer	Position ^a	Seq. (5'-3') ^b	T _m (°C) ^c	Target gene	Reference
27F	8 – 25	AGA GTT TGA TCM TGG CTC	46 - 48	16S rRNA, <i>Bacteria</i>	Lane 1991
27F-IRD700 ^d	8 – 25	AGA GTT TGA TCM TGG CTC	46 - 48	16S rRNA, <i>Bacteria</i>	Lane 1991
907RM	926 – 907	CCG TCA ATT CMT TTG AGT TT	46 – 48	16S rRNA, universal	Lane 1991
A364F	364 – 344	CGG GGY GCA SCA GGC GCG AA	62 – 64	16S rRNA, <i>Archaea</i>	Burggraf et al. 1997
A934R-IRD700 ^d	914 – 934	GTG CTC CCC CGC CAA TTC CT	58	16S rRNA, <i>Archaea</i>	Grosskopf et al. 1998
M13uniF	n.a.	TGT AAA ACG ACG GCC AGT	48	pGEM-T Multiple Cloning Site	Messing 1983
M13revR	n.a.	CAG GAA ACA GCT ATG ACC	48	pGEM-T Multiple Cloning Site	Messing 1983

^a Numbers in the names of primers indicate the 5'-position of binding sites relative to the reference sequence of *Escherichia coli* [ACCX80725].

^b Y, C or T; M, A or C; S, G or C (IUPAC, International Union of Pure and Applied Chemistry).

^c T_m (basic melting temperature) of primers was calculated online with OligoCalc: <http://www.basic.northwestern.edu/biotools/oligocalc.html>; last visit: 15.07.2010; Kibbe 2007.

^d Primer was labeled with the infra-red dye IRD700 at the 5' end.

n.a. Non-applicable.

F, Forward primer.

R, Reverse primer.

PCR reactions were prepared in 25, 50 or 100 µl volumes (according to requirements) with reagents of 5Prime (Hamburg, Germany), Biatec (Viernheim, Germany) and Eppendorf AG (Hamburg, Germany) (Table 12).

Table 12. Chemical composition of PCR reactions (reaction volume: 25 μ l, 1 reaction).

Reagents (Conc.)	16S rRNA gene PCR		M13-PCR	
	Volume [μ l]	Final conc.	Volume [μ l]	Final conc.
5Prime-Mastermix (2.5 x) ^a	10	1 x	-	-
Mg ²⁺ (25 mM)	1	1 mM	2.5	2.5 mM
Forward primer ^b (10 μ M)	1.5	0.6 μ M	0.5	0.2 μ M
Reverse primer ^b (10 μ M)	1.5	0.6 μ M	0.5	0.2 μ M
PCR-Buffer B (10 x) ^c	-	-	2.5	1 x
dNTP-mix (2 mM; Eppendorf)	-	-	5	0.2 mM
Crystal <i>Taq</i> -DNA- Polymerase (5 U μ l ⁻¹ ; Bilatec)	-	-	0.1	0.02 U μ l ⁻¹
Template DNA or cDNA	8.5	-	1 pipet tip cell material	-
PCR-H ₂ O	Ad 25	-	Ad 25	-

^a 5Prime Mastermix (2.5 x; 5Prime, Hamburg, Germany) contains *Taq* DNA Polymerase (62.5 U ml⁻¹), 125 mM KCl, β -CA360 in 0.5%, 500 μ M of each dNTP, 75 mM Tris-HCl [pH 8.3], 3.75 mM Mg²⁺, 0.25% Igepal and stabilizers.

^b Details about primers in Table

11.

^c PCR Buffer B (10 x; Bilatec, Viernheim, Germany) contains 0.2 M (NH₄)₂SO₄, 0.8 M Tris-HCl (pH 9.4 – 9.5), 0.2% (w/v) Tween-20.
conc., Concentration.

Thermal protocols for amplification of 16S rRNA genes and clone insert sequences are summarized in Table 13. PCR-H₂O was used as negative control, DNA of *Escherichia coli* (X80725) or *Methanosarcina barkeri* (AJ012094) was used as positive control for bacterial or archaeal 16S rRNA gene amplification, respectively. Quality of the PCR reactions was always checked by agarose gel electrophoresis (2.5.5).

Table 13. Thermal protocols for amplification of 16S rRNA genes and clone insert sequences.

No.	Step	Primers pairs ^a		
		27F ^b / 907RM	A364F / A934R-IRD700	M13uniF / M13revR
		T (°C) / time (minutes:seconds)		
1	Initial denaturation	95 / 5:00	95 / 5:00	95 / 10:00
	Step 2 – 4	4 x		
2	Denaturation	95 / 1:00	-	-
3	Annealing	40 / 1:00	-	-
4	Elongation	72 / 1:30	-	-
	Step 5 – 7	30 x	32 x	35 x
5	Denaturation	95 / 1:00	95 / 0:35	95 / 0:30
6	Annealing	50 / 0:30	60 / 0:30	50 / 0:30
7	Elongation	72 / 1:30	72 / 0:45	72 / 1:30
8	Final Elongation	72 / 10:00	72 / 7:00	72 / 10:00
Length of amplicon		~920 bp	~590 bp	~1070 bp

^a Details about primers in Table 11.

^b Primer was used in the labeled form(27F-IRD700) for tRFLP analysis.
bp, Base pairs.

2.5.9.2. 16S/18S rRNA Genes

16S rRNA genes of *Bacteria* and *Archaea* were amplified in cDNA samples derived from the 'light' and 'heavy' fraction of stable isotope probing experiments (2.3.1) to perform tRFLP fingerprinting analyses (2.5.11). Amplification of bacterial 16S rRNA genes was performed with the primer pair 27F/907RM (Table 11) of which the forward primer was either labeled (for tRFLP analyses) or unlabeled (for gene libraries). PCR products were about 920 bp long (Table 13). Amplicons of archaeal 16S rRNA genes were achieved with the primer pair A364F/A934R-IRD700 (Table 11) and had a length of about 590 bp (Table 13). Bacterial 16S rRNA genes derived from the 'heavy' fraction were used to construct gene libraries (2.5.12).

18S rRNA genes of *Eukarya* were amplified in cDNA samples of cellulose-supplemented stable isotope probing experiments at the Helmholtz Center for Environmental Research (UFZ) in Leipzig, Germany. Ciliate-specific primers were mixed (Table 14). All analyses including cloning, sequencing, and tRFLP were performed by the working group of Dr. Antonis Chatzinotas (Department of Ecological Microbiology, UFZ) and not further described.

Table 14. Primers for amplification of 18S rRNA genes of *Eukarya*.

Name of Primer	Seq. (5'-3') ^a	T _m (°C) ^b	Target gene	Reference
Cil_F	TGG TAG TGT ATT GGA CWA CCA	50.5	Ciliates	Lara et al. 2007
Cil_Ra	TCT GAT CGT CTT TGA TCC CTT A	51.1	Ciliates	Lara et al. 2007
Cil_Rb	TCT RAT CGT CTT TGA TCC CCT A	51.1 – 53.0	Ciliates	Lara et al. 2007
Cil_Rc	TCT GAT TGT CTT TGA TCC CCT A	51.1	Ciliates	Lara et al. 2007
Euk20F	GTA GTC ATA TGC TTG TCT C	46.8	universal	White et al. 1990
Euk516R	ACC AGA CTT GCC CTC C	48.5	universal	Amann et al. 1990
Bodo_80F	CAT CAG ACG YAA TCT GCC GC	53.8 – 55.9	<i>Bodonidae</i>	Unpublished
Bodo_651R	TTG GTC GCR CTT YTT TAG TCA CAG	54.0 – 57.4	<i>Bodonidae</i>	Unpublished
Spum_240F	GGA AAC CAA TGC GGG GCA AC	55.9	<i>Spumella</i> -like flagellates	Unpublished
Spum_651R	CTA TTT TGC TCA CAG TAA ATG ACG AG	54.8	<i>Spumella</i> -like flagellates	Unpublished

^a R, A or G; W, A or T; Y, C or T (IUPAC, International Union of Pure and Applied Chemistry).

^b T_m (basic melting temperature) of primers was calculated online with OligoCalc:

<http://www.basic.northwestern.edu/biotools/oligocalc.html>; last visit: 15.07.2010; Kibbe 2007.

F, Forward primer.

R, Reverse primer.

2.5.9.3. Clone Insert Sequences

M13-PCR was used (Table 12) to determine if clones (2.5.12.3) were positive, i.e., clones carry pGEM-T vectors with insert sequence of the expected length (in regard to the target gene). Cell material was directly picked from colonies and used as template for amplification of the inserted genes with the primer pair M13uniF/M13revR (Table 11). These primers target the flanking regions of the multiple cloning site (MCS) of the pGEM-T vector (Sambrook and Russell 2001; Yansich-Perron et al. 1985). The desired length is calculated by addition of ~150 bp to the length of the appropriate gene, i.e., insertion of the 920-bp long bacterial 16S-rRNA amplicon leads to a length of ~1,070 bp the M13-amplicon (Table 13). M13-PCR products were purified (2.5.3.3), sequenced (2.5.13), and further processed for phylogenetic analyses (2.5.14).

2.5.10. Quantitative PCR (qPCR)

Quantitative PCR (qPCR) is a highly sensitive tool for quantification of genes in a variety of samples (e.g., humans, marine or terrestrial ecosystems, etc.; Cikos and Koppel 2009; Heid et al. 1996; Raeymaekers 2000). A non-specific fluorescent dye that intercalates with double-stranded DNA (e.g. SybrGreen) is added to the PCR reaction. Intercalation with amplicons causes a strong fluorescence of the dye whose signal intensity increases relative to the amount of amplified target genes. The kinetic of PCR reactions is sigmoid, i.e., during the exponential phase the number of target molecules doubles with every PCR cycle. Parallel recording of the fluorescence signal of a DNA standard (with defined initial copy number) allows absolute quantification of the target molecules in environmental samples by comparison of the corresponding threshold cycles (C_T) (Figure 8). The C_T is the number of cycles at which the exponential phase begins and is correlated with the logarithm of the number of target molecules initially present in the reaction mixture (SQ, starting quantity). Half logarithmic plotting of standard C_T s against the corresponding SQ yields a linear regression, which can be used for quantification of the target gene in environmental samples. QPCR was performed with DNA and cDNA (RTqPCR; reverse transcription qPCR) derived from treatments with pesticide supplementation (2.3.3.3) and from treatments in self-constructed incubation chambers (2.3.2).

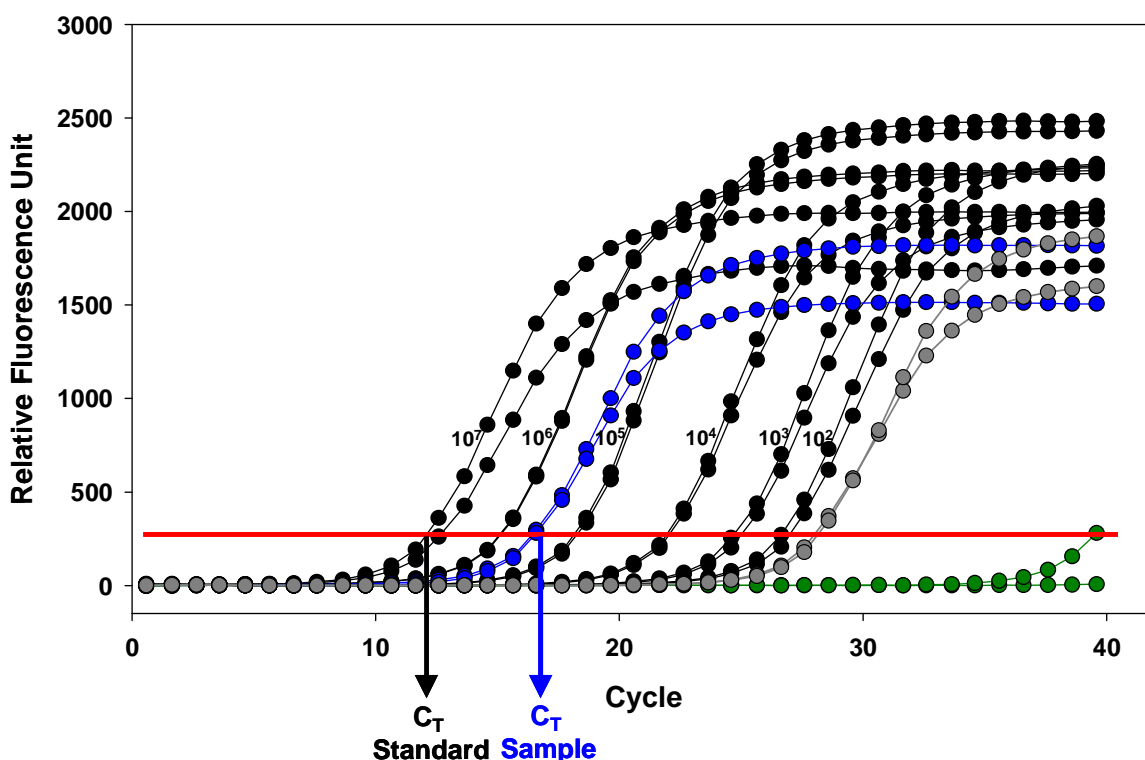


Figure 8. Determination of the threshold cycle (C_T) during quantitative PCR reaction. Non-target control, DNA with at least two mismatches to target gene (2.5.10.1; 3.4). ●, DNA standard (duplicated; $10^7 - 10^2$ copies reaction⁻¹; black); ●, Environmental sample (blue); ●, Non-template control, i.e., PCR-H₂O (green); ●, Non-target control, i.e., 10^5 copies reaction⁻¹ (grey); Red line, Fluorescence threshold.

2.5.10.1. QPCR Primers and Thermal Protocols

16S rRNA gene copy numbers of the domains *Bacteria* and *Archaea* were quantified according to previously published primers and protocols (Muyzer et al. 1993; Raskin et al. 1994; Table 15 - Table 17). Primers and assays for quantification of bacterial family-level taxa (Table 15) that were identified as active members of the total cellulolytic and saccharolytic microbial community (2.5.6, 2.5.11, 2.5.14) were designed and optimized in this study (3.4). All environmental DNA and cDNA samples were diluted 20- or 10-fold, respectively, to reduce potential inhibition of qPCR by co-extracted PCR-inhibiting compounds. Nonetheless, transcript numbers were corrected for inhibition by measuring inhibition factors for all samples (2.5.10.4; Table 15).

Table 15. Primers for quantification of 16S rRNA genes of *Bacteria*, *Archaea* and family-level taxa, and for inhibition correction by qPCR.

Name of Primer	Position ^a	Sequence (5'-3') ^b	T _m (°C) ^c	Target genes of	Reference
Domain-level Assays^d					
Gm5F	341 – 358	CCT ACG GGA GGC AGC AG	54	<i>Bacteria</i>	Muyzer et al. 1993
Eub534R	517 – 534	ATT ACC GCG GCT GCT GG	52	<i>Bacteria</i>	Muyzer et al. 1993
Arc344F	363 – 364	ACG GGG YGC AGC AG	54	<i>Archaea</i>	Raskin et al. 1994
Arch915R	915 – 934	GTG CTC CCC CGT AGG AGT	56	<i>Archaea</i>	Raskin et al. 1994
Family-level Assays^d					
Micro1R	596 – 613	GGA <u>CTT</u> TCA <u>CGG</u> CAG ACG	53	<i>Micrococcaceae</i> & <i>Cellulomonadaceae</i>	this work
Kin3R	620 – 635	CAG GCC <u>CGG</u> GGT <u>TAA</u>	47	<i>Kineosporiaceae</i> & <i>Nocardioideaceae</i>	this work
CloSac1R	838 – 852	<u>AGA</u> GGT CAT <u>GAC</u> AAC	39	Cluster I <i>Clostridiaceae</i>	this work
CloCel2R	480 – 495	TTT CCT CTC <u>CAG</u> CAC <u>TCA</u> A	49	Cluster III <i>Clostridiaceae</i>	this work
Pla4F	100 – 120	GGG <u>AAC</u> <u>CGG</u> CGT AAG GGG C	60	<i>Planctomycetaceae</i>	this work
PlaGm5R	341 – 357	CTG CAG CCA CCC GTG G	54	<i>Planctomycetaceae</i>	this work
Cel4R	488 – 505	CGA TGC TTA TTC <u>ATA</u> CAC	44	'Cellu1-3' ^e	this work

Name of Primer	Position ^a	Sequence (5'-3') ^b	T _m (°C) ^c	Target genes of	Reference
Sph1R	670 – 685	<u>CTG</u> TCA ATT CCG CCT	42	'Sphingo1-4' ^e	this work
Deh1R	580 – 600	<u>CGA</u> CTT <u>GAA</u> CGA CCG CCT	53	'Deha1' ^e	this work
DNA Inhibition Correction Assay					
T7PromF	n.a.	TAA TAC GAC TAT AGG G	38	pGEM-T vector insert	Degelmann et al. 2009b
M13RevR	n.a.	CAG GAA ACA GCT ATG ACC	48	pGEM-T vector insert	Degelmann et al. 2009b
RNA Inhibition Correction Assay					
lhcF	n.a.	ATT GGG CCC GAC GTC	47	pGEM-T vector insert	Wieczorek et al. 2011
lhcR	n.a.	ATT TAG GTG ACA CTA TAG AAT A	46	pGEM-T vector insert	Wieczorek et al. 2011

^a Numbers in the names of primers indicate the 5'-position of binding sites relative to the reference sequence of *Escherichia coli* [ACCX80725]. Abbreviations: F, forward primer; R, reverse primer.

^b Y, C or T (IUPAC, International Union of Pure and Applied Chemistry).

^c T_m (basic melting temperature) of primers was calculated online with OligoCalc: <http://www.basic.northwestern.edu/biotools/oligocalc.htm>; last visit: 15.07.2010; Kibbe 2007

^d Bold and underlined bases in primer sequence of family-level taxa indicate mismatches to non-target control (3.4).

^e Names and affiliation of family-level taxa according to (3.2.4.2).

n.a., Non-applicable.

Primers used in qPCR reactions (Table 15) were purchased from Biomers GmbH (Biomers.net, Ulm, Germany). PCR reactions were prepared in 20 µl volumes with PCR chemicals of Bioline (Luckenwalde, Germany) (Table 16) and performed on an iQ5 iCycler (Bio-Rad, Hercules, USA).

Table 16. Chemical composition of qPCR reactions.

Reagents (Conc.)	Domain-level assays		Family-level assays		Inhibition correction assays	
	Volume [μ l]	Final conc.	Volume [μ l]	Final conc.	Volume [μ l]	Final conc.
SensiMix SYBR & Fluorescein (2x) ^a	10	1 x	10	1 x	10	1 x
Forward primer ^{b, c} (10 μ M)	1.5	0.75 μ M	1 – 1.5	0.5 – 0.75 μ M	1	0.5 μ M
Reverse primer ^{b, c} (10 μ M)	1.5	0.75 μ M	1.5 – 3	0.75 – 1.5 μ M	1	0.5 μ M
Template DNA or cDNA	5.0	-	5.0	-	5.0	-
PCR-H ₂ O	Ad 20	-	Ad 20	-	Ad 20	-

^a SensiMix SYBR & Fluorescein (2 x; Biorline, Luckenwalde, Germany) contains reaction buffer, heat-activated DNA Polymerase, ultra-pure dNTPs, 6 mM MgCl₂, stabilizers, Fluorescein, and SYBR® Green I.

^b Details about primers in Table 15.

^c The final concentration of each primer in family-level assays number 1, 3, 5 and 8 was 750 nM (Table 25; 3.4). The final concentration of each primer in family-level assays number 2, 4, 6 and 7 was 500 nM (forward primer) and 1500 nM (reverse primer) (Table 25; 3.4).
conc., Concentration

Thermal protocols are summarized in Table 17. Details about family-level assays are given in the results section (3.4). The fluorescence signal was recorded during elongation. Subsequent agarose gel electrophoresis (2.5.5) was used to check for the formation of unspecific bands. Specificity of domain- and family-level assays was analyzed by sequencing of qPCR amplicons (2.5.10.2).

Table 17. Thermal protocols for amplification of 16S rRNA genes and clone insert sequences.

		Primers pairs ^a				
		Family-level assays ^b	Gm5F / Eub534R	Arc344F / Arch915R	IhcF / IhcR	T7PromF / M13Rev
No.	Step	T (°C) / time (minutes:seconds)				
1	Initial denaturation	94 / 8:00	94 / 8:00	94 / 8:00	94 / 8:00	94 / 8:00
	Step 2 – 4	35 x	35 x	45 x	38 x	38 x
2	Denaturation	95 / 0:30	95 / 0:30	95 / 0:30	95 / 0:30-	95 / 0:30-
3	Annealing	56 – 67.5 ^b / 0:40	55.7 / 0:40	55.7 / 0:40	60 / 0:15	61.2 / 0:15
4	Elongation	72 / 0:40	72 / 0:30	72 / 0:30	72 / 0:45	72 / 0:45
5	Final Elongation	72 / 5:00	72 / 5:00	72 / 5:00	72 / 7:00	72 / 10:00
6	Melting curve		161 x 70 – 94 / 0:10 (+0.15°C / cycle)		49 x 70 – 94 / 0:10 (+0.5°C / cycle)	
Length of amplicon (bp)		See Table 25	190	570	450	450

^a Details about primers in Table 15.

^b Details about primer pairs, appropriate annealing temperature, and length of amplicon of family-level assay in the results section (3.4).

bp, Base pairs.

No., Number.

2.5.10.2. Preparation of Quantitative DNA Standards

Each qPCR was calibrated with quantitative assay-specific DNA standards. These standards were prepared with family-level 16S rRNA gene sequences re-amplified (primer pair: 27F/907RM, Table 11) from the corresponding clone insert sequence (2.5.9.3, 3.4). PCR products were purified with Millipore PCR₉₆ Cleanup Plates (Millipore Cooperation, Bedford, USA) (2.5.3.3) and quantified with the QuantiT-PicoGreen dsDNA reagent Kit (Invitrogen, Karlsruhe, Germany) (2.5.4.2). The number of target sequences c_{TS} was calculated with Equation 7, including the concentration of purified standard DNA, the length (i.e., the number of bases) of the amplicon, the molecular weight of one base pair in double stranded DNA, and the Avogadro constant N_A .

Equation 7. Number of target molecules in purified standard solutions.

$$c_{TS} = \frac{c_{DNAstd}}{N_{bases} \cdot MW_{bp}} \cdot N_A$$

c_{TS} , number of target sequences (targets μl^{-1}); c_{DNAstd} , concentration of standard DNA ($\text{ng}_{DNA} \mu\text{l}^{-1}$); N_{bases} , length of the amplicon; MW_{bp} , molecular weight of one base pair in double-stranded DNA ($MW_{bp} = 650 \text{ ng nmol}^{-1}$); N_A , Avogadro constant ($N_A = 6.23 \times 10^{23} \text{ molecules nmol}^{-1}$).

DNA standards were diluted with DEPC- H_2O to 2×10^9 target molecules μl^{-1} and stored at -80°C . Directly before use, dilution series were prepared to achieve concentrations of $10^7 - 10^0$ molecules per qPCR reaction by application of $5 \mu\text{l}$ of diluted standard DNA as template (2.5.10.1). DNA standards for quantification of *Bacteria* and *Archaea* were supplied by Katharina Palmer, Department of Ecological Microbiology, University of Bayreuth.

2.5.10.3. Evaluation of the Specificity of Assays in Soil Samples

Several factors were taken into account to evaluate target group specificity of family-level and domain-level qPCR assays. Melt curves of PCR products of qPCR standards were compared with PCR products amplified from soil samples. Subsequently, the formation of unspecific bands was checked by agarose gel electrophoreses (2.5.5). QPCR products from each assay of every sample (i.e., all replicates, time points, and substrates) of treatments in self-constructed incubation chambers (2.3.2) were pooled and sent to LGC Genomics (Berlin, Germany) for cloning and sequencing (2.5.13). 17 – 24 sequences per assay were analysed with BLAST (Altschul et al. 1990; 2.5.14).

2.5.10.4. Correction of PCR Inhibition and Calculation of Transcript Numbers

Co-extracted compounds (e.g. humic acids) or variable reverse transcription efficiencies may impact on qPCR measurements. This might lead to underestimation of transcript numbers (Degelmann et al. 2009b; Wieczorek et al. 2011). Thus, transcript numbers were corrected by measuring inhibition factors in all samples (Degelmann et al. 2009b; Wieczorek et al. 2011). A *pheA* (gene that encodes a phenol hydroxylase) containing pGEM-T vector insert was used as inhibition control DNA and as template for preparation of control RNA (2.5.8). Every environmental DNA and RNA sample was spiked with 2×10^9 inhibition control molecules μl^{-1} . Spiked RNA extracts were transcribed into cDNA (2.5.7), and all extracts were quantified with domain-, family-level, and DNA or RNA inhibition assays (Table 15 and Table 17). Corrected 16S rRNA gene transcript numbers ($\lg[\text{SQ}_{corrected}]$) of *Bacteria*, *Archaea* and family-level taxa were calculated with Equation 8.

Equation 8. Correction of transcript numbers by inhibition correction assays.

$$\lg(SQ_{corrected}) = \lg \left(SQ_{measured} \cdot \frac{\lg(SQ_{INHIBCORR_set})}{\lg(SQ_{INHIBCORR_is})} \cdot \frac{E_{Assay}}{E_{INHIBCORR}} \right)$$

$\lg(SQ_{corrected})$, corrected logarithmic values of the starting quantity (SQ) measured in domain- or family-level assays; $SQ_{measured}$, starting quantity measured in domain- or family-level assays; $\lg(SQ_{INHIBCORR_set})$, logarithmic values of the starting quantity of control DNA or RNA spiked into environmental extracts; $\lg(SQ_{INHIBCORR_is})$, logarithmic values of the starting quantity of control DNA or RNA measured; E_{Assay} , amplification efficiency (E) of domain- or family-level assay; $E_{INHIBCORR}$, amplification efficiency of the Inhibition correction assays.

Family-level assays (3.4) did not yield sufficient gene amplification in DNA samples (i.e., target sequences were below the detection limit of about 10 genes per reaction; $C_T > 30$). Presented transcript numbers in this study refer exclusively to transcript numbers quantified in cDNA samples. The total amount of RNA that was extracted from experimental replicates was similar, but variable (Figure 9). Thus, corrected 16S rRNA transcript numbers were quantified per ng RNA rather than per gram dry weight of soil. These absolute numbers of transcripts of family-level taxa were divided by the corresponding number of transcripts of total *Bacteria* to further correct for variability in lysis efficiencies and the growth of *Bacteria*. For each replicate, these copy numbers of family-level taxa were normalized based on the highest copy number measured (i.e., the highest number obtained for each assay, including substrate-supplemented and unsupplemented microcosms, was set at 100%) and are given in percent. Data presented are means of experimental triplicates (including standard deviations) or duplicates (without standard deviation).

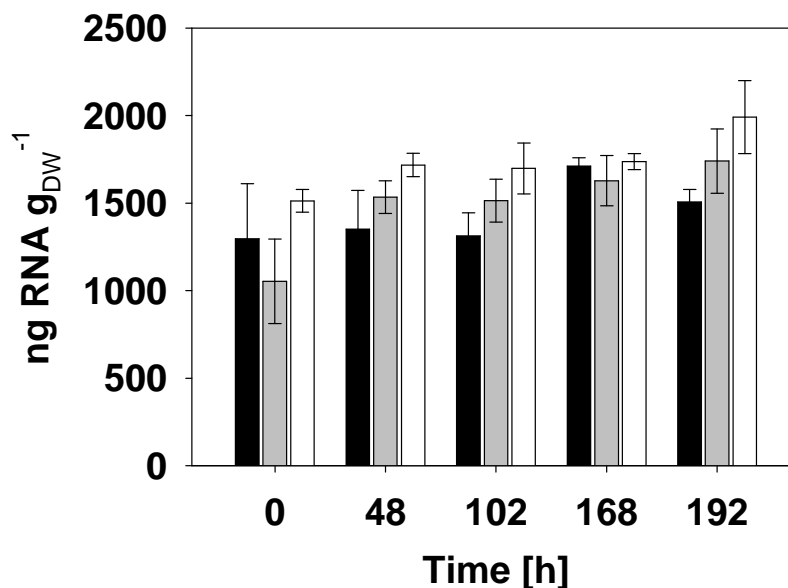


Figure 9. Total RNA extracted from each sampling point of each treatment. Filled bars, CMC-supplemented treatments; Shaded bars, cellobiose-supplemented treatments; Empty bars, unsupplemented controls. Error bars indicate standard deviations (n = 3).

2.5.11. Terminal Restriction Fragment Length Polymorphism (tRFLP) Analysis

TRFLP is a PCR-based fingerprinting method that allows for the rapid characterization of microbial communities in various environments (Liu et al. 1997; Thies 2007). 16S rRNA or functional genes are amplified with fluorescence labeled primers (one or both), resulting in terminal-labeled PCR products (Figure 10). Digestion of these PCR products with a restriction endonuclease (type II) yields terminal-labeled restriction fragments (tRFs). These fragments are subsequently length separated on a polyacrylamide gel (PAGE) (Liu et al. 1997). Different microbial communities result in different tRF profiles in which every tRF is indicative for at least one community member (Marsh 2005). *In silico* digestion of ribosomal database sequences or clone insert sequences obtained from the same environmental sample allows the affiliation of single tRFs to microbial taxa.

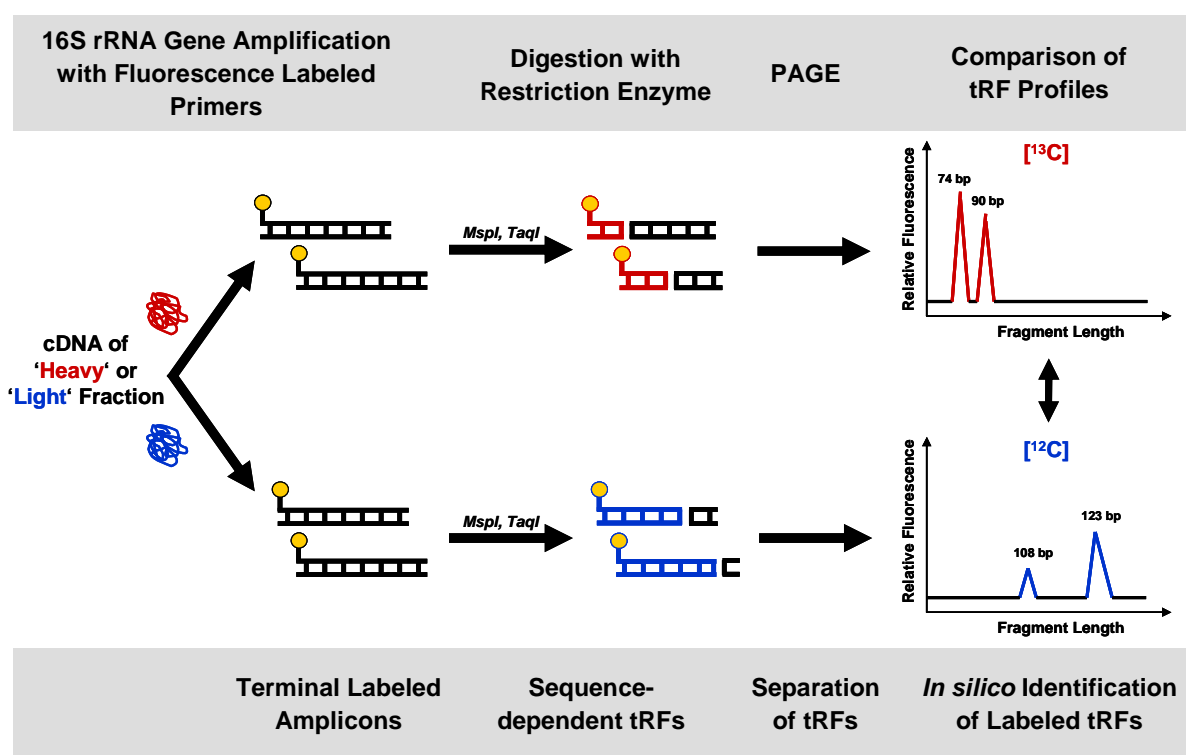


Figure 10. Scheme of tRFLP analysis. See text for details.

In this study, 16S rRNA cDNA gene based tRFLP was performed to rapidly compare bacterial and archaeal soil communities involved in the consumption of supplemented carbon (i.e., cellulose, cellobiose, glucose; 2.5.6). TRF profiles were further used to identify sequences of [¹³C]-labeled organisms in gene libraries. Although cDNA of 'heavy' fractions was used for cloning, unlabeled genotypes (i.e., 16S rRNA cDNA sequences) in 'heavy' fractions had high G+C contents (53 – 61%, data not shown) and may have thus been unfolded RNA molecules yielding buoyant densities similar to labeled genotypes (Lüders et al. 2004). Therefore, tRF profiles of *Bacteria* and *Archaea* were generated from cDNA samples derived from the 'light' and 'heavy' fraction of [¹²C]- and [¹³C]-stable isotope probing gradients (2.5.6.3, 2.5.7), and compared. This minimizes overestimation of the active part of the

microbial community. TRF profiles of day 0 (corresponding to the autochthonous soil community) were generated exclusively for [¹³C]-treatments, since the same homogenized soil samples was used in [¹²C]-treatments. Primer pairs, PCR conditions, and thermal protocols as mentioned above (Table 11 - Table 13).

2.5.11.1. Mung Bean Endonuclease Digestion

Prematured termination of the elongation step during PCR can lead to single-stranded parts of the amplicons. This, in consequence, might promote the formation of 'pseudo-tRFs', i.e., tRFs that result from false restriction digestion at non-terminal restriction sites (Egert and Friedrich 2003). Single-stranded extensions at the terminal ends were removed with mung bean endonuclease digestion (New England Biolabs, Ipswich, USA). 50 µl of purified PCR product were mixed with 5.5 µl reaction buffer NEB2 (10x) and 2 µl mung bean endonuclease (0.5 U µl⁻¹). Incubation was performed at 30°C for 1 hour. Endonuclease digestion was stopped by purification of samples with Millipore PCR₉₆ Cleanup Plates (Millipore Cooperation, Bedford, USA) (2.5.3.3).

2.5.11.2. Restriction Digestion

Subsequent restriction digestion was performed with *MspI* (5' → 3' restriction site: C[^]CGG; New England Biolabs, Ipswich, USA, bacterial 16S rRNA genes) or *TaqI* (5' → 3' restriction site: T[^]CGA; New England Biolabs, USA; archaeal 16S rRNA genes) (Degelmann et al. 2009b). 7 µl purified PCR product were mixed with 1 µl reaction buffer NEB2 (10x; *MspI*) or 1 µl NEB3 (10x, *TaqI*), 1 µl BSA (10x), and 1 µl restriction enzyme (2 U µl⁻¹). Restriction digestion was performed at 37°C for 4 hours (*MspI*) or at 65°C for 3 hours (*TaqI*). Reactions were stopped by heat inactivation of the enzyme for 5 minutes at 95°C (*MspI*) or 20 minutes at 80°C (*TaqI*). Remaining DNA was quantified with the Quant-iT-PicoGreen dsDNA reagent Kit (Invitrogen, Karlsruhe, Germany) (2.5.4.2) and diluted with PCR-H₂O to a concentration of 0.5 ng µl⁻¹.

2.5.11.3. Denaturing Polyacrylamide Gelelectrophoresis (PAGE)

Denaturing gel electrophoresis was performed on a NEN model 4300 DNA analyzer (Licor, Lincoln, USA) as described (Hamberger et al. 2008). Gel plates (Borofloat glass plates 25 mm x 25 mm x 5 mm, LICOR, Lincoln, USA) were thoroughly cleaned sequentially with ddH₂O, ethanol (70%) and isopropanol (80%). For stabilization of the gel pockets 250 µl bind-silane solution (1:1 bind silane [plusOne; GE Healthcare, Piscataway, USA] and 10% CH₃COOH) was applied to the plates at the area at which the comb has to be inserted. The plates were separated by spacers (0.2 mm tick), fixed with assembly rails and casting plate, and placed in the gel casting stand. The polyacrylamide gel was prepared by gently mixing 15 g urea (Roche Pharma, Reinach, Switzerland), 3.75 ml of 40% acrylamide-bis solution (37.5 : 1; 2.6% C; Bio-RAD, Hercules, USA), 6 ml 5 x TBE buffer (450 mM Tris, 450 mM H₃BO₃, 10 mM EDTA, pH 8 [Sambrook and Russell 2001]), and 9.25 ml ddH₂O. The gel solution was filtered (Ø 0.2 µm) to remove excess undissolved salts. The polymerisation reaction was started by addition of 175 µl ammonium persulfate (APS, 440 mM) and 17 µl N,N,N,N-Tetramethylethylenediamine (TEMED, ultra-pure; Invitrogen, Karlsruhe, Germany). The gel was poured and a rectangular tooth comb (48 lanes) was inserted. Polymerisation was carried out at 25°C for 45 minutes. The

glass plates were placed into the DNA analyser, the buffer tanks were fixed, and filled with 1 x TBE buffer. The comb was removed and gel pockets were cleaned from precipitated urea by gently flushing with buffer. Before samples were loaded onto the gel a pre-run was performed at 1,200 V and 45°C for 25 minutes. Samples were prepared for electrophoresis by mixing 2 µl of sample with 2 µl Stop-Solution (Licor, Lincoln, USA), denaturation at 95°C for 3 minutes, and placing on ice. A 50 – 700 bp length standard (Size Standard IRDye 700; Licor Biosciences or microSTEP-24a (700); Microzone Ltd, Haywards Heath, UK) was processed analogous. 0.5 µl size standard or 0.8 µl sample were loaded onto the gel and electrophoresis was performed at 1,200 V and 45°C for 4 hours.

2.5.11.4. Analysis of tRFLP Profiles

Scanned gel images were exported and analysed with GelQuest (Version 2.6.3.; Sequentix, Klein Raden, Germany). This program generates tRFLP profiles in which every peak represents one tRF. The peak height of a tRF is directly linked to the relative intensity of the fluorescence label. To compare relative numbers, the fluorescence value of a detected tRF was normalized against the fluorescence value of the respective tRF in the tRFLP profile with the lowest total fluorescence. TRFs with values below 5% were excluded. Means and standard deviations were calculated for t_{end} measurements of [^{13}C]-supplemented treatments (2.3.1). The tRFLP profiles derived from 'heavy' RNA fractions of [^{13}C]- and [^{12}C]-treatments were compared for identifying tRFs of labeled genotypes. The tRFs were scored as labeled when they were present only in [^{13}C]-profiles or when the relative intensity in [^{13}C]-profiles was higher than in [^{12}C]-profiles at the same respective time interval. This procedure avoided an overestimation of labeled genotypes, as unlabeled genotypes may co-migrate towards the 'heavy' RNA fraction (Lüders et al. 2004; Manefield et al. 2002a; Rangel-Castro et al. 2005). TRFs were assigned to putative taxa by *in silico* analyses of clone insert sequences (2.5.12) and ribosomal databases using the software tool TRFCUT included in ARB (Ricke et al. 2005; 2.5.14.3).

2.5.12. Construction of Gene Libraries

Gene libraries are constructed by insertion of foreign DNA (e.g. PCR products of certain genes) into a cloning vector (ligation) and further introduction of this vector (transformation) into competent host cells (Sambrook and Russell 2001; Yansich-Perron et al. 1985). Cultivation of host cells yields clones (i.e., colonies) of which the ones are picked that apparently carry cloning vectors with inserted DNA (blue/white screening). 16S rRNA cDNA genes of each *Bacteria*, *Archaea*, and family-level taxa amplified by qPCR (2.5.10, 2.3.2) were pooled (i.e., all replicates, time points, and substrates). Cloning of bacterial 16S rRNA genes amplified from cDNA of the 'heavy' fraction of [^{13}C]-gradients (2.3.1, 2.5.9.2) was performed with the pGEM-T vector system II (Promega, Madison, USA) after the manufacturer's protocol.

2.5.12.1. Ligation

The pGEM-T vector (size: ~3000 bp) is linearized with a single 3'-terminal thymidine at both ends. This prevents recircularization of the vector and improves the efficiency of ligation of the PCR product into the vector (due to a polymerase-mediated adenosine-overhang at the 3' end of the DNA strand; Mezei and Storts

1994; Robles and Doers 1994). The vector contains a gene for ampicillin-resistance and a MCS (multiple cloning site) that is located in the *lacZ* operon. *lacZ* encodes for the enzyme β -galactosidase and its operon is disrupted by insertion of a foreign DNA at the MCS. Thus, *lacZ* cannot be expressed in host cells that took up a plasmid with DNA insert. The inability of such clones to produce β -galactosidase can be used for selection of insert positive clones (2.5.12.3). The optimal insert to vector molar ratio for the pGEM-T vector system is denoted to be 1:1, but successful ligation can also be achieved between ratios of 8:1 and 1:8. The appropriate amount of insert to include in the ligation reaction can be calculated for different ratios (Equation 9). A ratio between 1:1 and 3:1 was used in this study, what corresponds to 15 – 45 ng of PCR product per reaction (Table 18).

Equation 9. Insert : vector ratio.

$$ng_{insert} = \frac{ng_{vector} \cdot kb \text{ size of insert}}{kb \text{ size of vector}} \cdot \text{insert : vector molar ratio}$$

Purified 16S rRNA cDNA gene PCR products (2.5.3.2) were inserted into the vector plasmid (pGEM-T) by a T4 DNA ligase. The ligation reactions were prepared (Table 15), mixed, placed into a water bath (25°C), and incubated overnight at 4°C. The optimal temperature for ligation was perambulated during the cool down of the water bath.

Table 18. Ligation reaction mix.

Reaction component (concentration)	Amount (μ l)	Final concentration
Rapid Ligation Buffer (2 x) ^a	5	1x
pGEM-T vector (50 ng μ l ⁻¹)	1	5 ng μ l ⁻¹
T4 DNA Ligase (3 Weiss U μ l ⁻¹)	1	0.3 U μ l ⁻¹
PCR product (insert DNA)	0.5 - 2	15 – 45 ng
PCR-H ₂ O	Ad 10	-

^a 2x Rapid Ligation Buffer contains 60 mM Tris-HCl (pH 7.8), 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol (MW8000, ACS Grade).

2.5.12.2. Transformation

Vector plasmids were inserted into high-efficiency competent *Escherichia coli* JM 109 cells ($> 10^8$ cfu μ g_{DNA}⁻¹). *E. coli* cells were purchased from Promega (Madison, USA) or prepared by Ralf Mertel at the Department for Ecological Microbiology (University of Bayreuth) according to the following procedure.

Liquid *E. coli* cultures (in glycerol) were transferred onto LB-plates and incubated at 37°C for 12 hours. One colony was picked, transferred into 5 ml LB-medium (2.1) and incubated overnight. 1 ml overnight-culture was used as inoculum for 50 ml LB-medium and cultures were incubated at 37°C for about 5 hours until an optical density (OD₆₆₀) of 0.5 was reached. The cells were collected by centrifugation (4,000 rpm, 4°C, 5 minutes), resuspended in 15 ml sterile, ice-cold transformation buffer 1 (10 mM CaCl₂, 50 mM MnCl₂ · 4 H₂O, 30 mM potassium acetate [CH₃CO₂K], 100 mM RbCl, 15% glycerol, pH 5.8), and incubated on ice for 90 minutes. Subsequently, cells were collected by centrifugation and resuspended in 2 ml sterile, ice cold transformation buffer 2 (75 mM CaCl₂, 10 mM MOPS, 10 mM RbCl, 15% glycerol, pH 8.0). Cells were stored in 220 µl portions at -80°C. Directly before use cells were thawed on ice.

For transformation 220 µl competent cells were gently mixed with 2.5 µl ligation reaction and mixtures were incubated on ice for 30 minutes. Cells were placed in a preheated water bath (42°C) for 45-50 seconds (heat-shock) to force uptake of the vector plasmid. The cells were immediately placed on ice for 2 minutes. 950 µl SOC-medium (2.1) were added and cells were incubated at 37°C for 90 minutes on a thermomixer (Eppendorf, Hamburg, Germany) under constant slow shaking (300 rpm). Subsequently, about 400 µl of transformed cells were spread over LB/Ampicillin/IPTG/X-Gal-plates (2.1) and incubated overnight at 37°C. The addition of ampicillin to agar plates exclusively allows growth of cells that acquired the ampicillin-resistance gene by uptake of the vector plasmid.

2.5.12.3. Blue/white Screening of Clones

Blue/white screening (Sambrook and Russell 2001) was used to select for clones that do not only carry a vector plasmid (as selected by ampicillin resistance), but also a plasmid with a DNA insert at the MCS. IPTG induces the expression of the *lac* operon and hence the production of the enzyme β-galactosidase. This enzyme reacts with the colourless compound X-Gal (lactose analogue) and leads to the formation of the blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo, causing a characteristic blue colour in the colonies. White colonies are sufficient for the production of β-galactosidase, indicating that the *lacZ* operon is disrupted by a DNA insert. White colonies were randomly picked with a sterile toothpick, transferred onto fresh LB/Ampicillin/IPTG/X-Gal-Plates (2.1), and incubated at 37°C overnight. Selection of positive clones that carry vector plasmids with putative 16S rRNA cDNA gene insert sequences was performed by amplification of the insert DNA by M13-PCR (2.5.9.3).

2.5.13. Sequencing

Purified M13-PCR products of 16S rRNA cDNA gene insert sequences (2.5.9.3) were sent to Macrogen (Kumchun-ku Seoul, Korea) for sequencing. QPCR products of domain- and family-level assays (2.5.10.3) were sent to LGC Genomics (Berlin, Germany) for cloning and sequencing. M13uniF (Table 11) was used as sequencing primer.

2.5.14. Gene Sequence Analysis

Mega 4 (Version 4.1 Beta; <http://www.megasoftware.net/>; last visit: 08.11.2011; Kumar et al. 2008b) was used to cut off vector residues and to arrange all sequences in the same direction (5'→3'). Sequences were further checked for the occurrence of chimeras with the online tool Ribosomal Database Project (RDP) Chimera Check 2.7 (<http://rdp8.cme.msu.edu/cgiis/chimera.cgi>; last visit: 20.10.2010; Cole et al. 2007; Cole et al. 2009). Chimeras are sequences that are composed of two or more fragments of phylogenetically different organisms. Such hybrid sequences can assemble during PCR (Acinas et al. 2005; Ashelford et al. 2006; Liesack et al. 1991). Chimeras might suggest the detection of non-existing genotypes and can lead to an overestimation of the total microbial diversity (Hugenholtz and Huber 2003; Smyth et al. 2010). Chimeric sequences were corrected by removing the shorter part of the sequence at the point at which the phylogenetic different fragments were connected. Sequences that were shorter than 350 bp, were excluded from the datasets. The online tool BLASTn (nucleotide blast; <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>; last visit: 08.11.2011; Altschul et al. 1990; Zhang et al. 2000) was applied to identify qPCR-derived sequences (2.5.10.3). QPCR-derived sequences were only used for evaluation of the specificity of qPCR assays by BLASTn and not further analysed. Sequences that were derived from stable isotope probing experiments were pre-aligned with the online tool SINA Webaligner (<http://www.arb-silva.de/aligner>; including the nearest neighbour option; last visit: 08.11.2011; Prüsse et al. 2007) and further processed with the ARB software package (Version 2005; <http://www.arb-home.de>; last update: 27.08.2011; Ludwig et al. 2004) on the basis of database release 104 of SILVA (<http://www.arb-silva.de>; last visit: 08.11.2011; Prüsse et al. 2007).

2.5.14.1. Processing of Sequences Derived from Stable Isotope Probing Experiments

The ARB included software tool TRFCUT (Ricke et al. 2005) was applied to assign clone insert sequences to labeled or unlabeled tRFs within the tRF profile of the corresponding sample (2.5.11.4). Sequences related to unlabeled tRFs were only analysed with BLASTn for their phylogenetic affiliation to microbial families (sequence similarity > 87%; Yarza et al. 2008; Yarza et al. 2010), but not further processed. Sequences that represented labeled tRFs were compared with DOTUR (Schloss and Handelsman 2005) and grouped into family-level OTUs (operational taxonomic unit), i.e. sequences that showed less than 13% distance were grouped into the same OTU (= taxon) (Yarza et al. 2008). DOTUR results were checked by calculation of distance matrices for the same datasets in ARB. OTUs were identified by BLASTn analysis of 1 - 4 representative sequences (dependent on the number of sequences in one OTU) and assigned to validated microbial families (Euzéby 2011). Due to different algorithms of DOTUR and BLASTn it is possible that several OTUs are assigned to the same family. Such OTUs were condensed in one family-level taxon.

The coverage of a gene library can be used as a qualifier to analyse how many of OTUs were detected in regard to the total number of estimated OTUs (Good 1958). Coverages (C) were calculated with Equation 10 (Schloss et al. 2004).

Equation 10. Coverage.

$$C = \left(1 - \frac{n}{N}\right) \cdot 100$$

n, number of OTUs containing a single sequence; N, total number of sequences analysed.

2.5.14.2. Rarefaction Analysis

Rarefaction analysis allow comparison of gene libraries with different sequence numbers by estimation of the total diversity that might occur in a certain sequence data set (Hartmann and Widmer 2006; Holland 2003). The number of expected OTUs (S_i) is represented as a function of the number of analysed sequences (Rarefaction curve). Flat or plateauing curves indicate that most of the estimated genotypes were covered by the gene library. Steep rarefaction curve suggest insufficient sampling, i.e., a greater diversity would have been covered by analyses of a greater number of sequences. Rarefaction curves were calculated with the freeware aRarefact (<http://www.uga.edu/~strata/software>; last visit: 08.11.2011) using the method of Hurlbert (Heck et al. 1975; Hurlbert 1971).

2.5.14.3. Calculation of Phylogenetic Trees

Phylogenetic trees were built with ARB (version 2005). Trees included representative sequences of labeled family-level OTUs (2.5.14.1) and reference sequences of corresponding families or close related uncultured species. The algorithms 'Maximum-Likelihood' (AXML + FastDNAml; 50% filter; Olsen et al. 1994; Stamatakis et al. 2004) and 'Neighbor-Joining' (Saitou & Nei 1987; substitution model: felsenstein [Felsenstein 1985]) were used. Trees were compared to confirm tree topology. Nodes that were identical in neighbour-joining were marked in maximum-likelihood trees with dots by use of the graphical software iGrafx Designer (Micrografx, Tualatin, USA). Number of valid positions was dependent on the position relative to the sequence of *Escherichia coli* (X80725) and the length of sequences included in the tree. Details on valid positions are included in the figure legends. The out group was *Methanosarcina barkeri* (AF028692).

2.5.14.4. Deposition of Sequences in Official Databases

Sequences were deposited at the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk/embl>; last visit: 08.11.2011; European Bioinformatics Institute, Cambridge, UK). Representative sequences of labeled OTUs (2.5.11.4, 2.5.14.1) were deposited under accession numbers FN433934 to FN434041. Accession numbers FN433987 to FN434041, FN433934 to FN433957 and FN433958 to FN433986 correspond to sequences amplified from cDNA samples of the 'heavy' fraction of [¹³C]-cellulose, [¹³C]-cellobiose, and [¹³C]-glucose supplemented treatments, respectively. Sequences that were derived from qPCR products (2.5.10.3) were deposited at EMBL under accession numbers FR773527 to FR773699.

2.6. Statistics

All statistical analyses, including linear regression, consumption rates of sugars, and the stability index (R^2) were performed with the software Excel 2002 (Microsoft, Redmond, USA).

2.6.1. Mean Value, Standard Deviation and Error Propagation

Arithmetic means (\bar{x}), standard deviations (S) and standard errors ($S_{\bar{x}}$) were calculated according to Equation 11 - Equation 13 (Precht et al. 2005; Sachs 1999). n indicates the number of considered values. The continuous index runs from i to 1.

Equation 11. Arithmetic mean.

$$\bar{x} = \frac{1}{n} \cdot \sum_{i=1}^n x_i$$

Equation 12. Standard deviation.

$$S = \sqrt{\frac{1}{n-1} \cdot \left[\sum_{i=1}^n (x_i - \bar{x})^2 \right]}$$

Equation 13. Standard error.

$$S_{\bar{x}} = \frac{S}{\sqrt{n}}$$

Error propagation occurs when means of distinct measurements are combined (Fenner 1931; Precht et al. 2005; Sachs 1999). Error propagation $S_{\bar{y}}$ was calculated with Equation 14 by addition or subtraction of means ($\bar{y} = \bar{x}_1 \pm \bar{x}_2$) and with Equation 15 by multiplication ($\bar{y} = \bar{x}_1 \cdot \bar{x}_2$) or division of means ($\bar{y} = \frac{\bar{x}_1}{\bar{x}_2}$).

Equation 14. Error propagation by addition or subtraction of means.

$$S_{\bar{y}} = \sqrt{(S_{\bar{x}_1})^2 + (S_{\bar{x}_2})^2}$$

Equation 15. Error propagation by multiplication or division of means.

$$S_{\bar{y}} = \sqrt{\left(\frac{S_{\bar{x}_1}}{\bar{x}_1}\right)^2 + \left(\frac{S_{\bar{x}_2}}{\bar{x}_2}\right)^2}$$

2.6.2. Test of Significance (t-Test)

Statistical tests were performed to compare mean transcript numbers measured by qPCR (2.5.10). Dependent t-Tests (paired t-Test; David and Gunnink 1997; Timischl 2000) were used to analyze pairwise whether mean copy numbers of two time points within one specific assay were statistically different. Variables used were: $n = 3$ to 6 ; degree of freedom $f_d = -1$; significance level $\alpha = 0.05$; variance $v = 2.9200$ ($n = 3$); $v = 2.0150$ ($n = 6$) (Timischl 2000). The t value was calculated with Equation 16. $t > v$ implies a statistically significant difference between a data point and its preceding value. Statistic differences are marked specifically in figures and tables.

Equation 16. Dependent t-Test.

$$t = \frac{1}{\sqrt{n}} \cdot \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(S_{x_1})^2 + (S_{x_2})^2}}$$

3. RESULTS

3.1. Degradation of [^{12}C]- and [^{13}C]-Cellulose, -Cellobiose, and -Glucose under Oxic and Anoxic Conditions

Degradation of crystalline cellulose cannot be measured by HPLC directly. However, addition of [^{12}C]- and [^{13}C]-cellulose stimulated production of carbon dioxide by approximately 50% under both oxic and anoxic conditions (data not shown), indicating that cellulose was degraded. The potential degradation of cellulose is further supported by the labeling of well-known cellulolytic taxa in cellulose-supplemented treatments (4.2, 4.2.2). Primary hydrolytic products of cellulose (i.e., cellobiose and glucose) were detected neither during oxic nor anoxic treatments (i.e., they were below the detection limit of 50 μM) what is already known from mixed and pure cultures in chemostats (Chyi and Dague 1994; Lynd et al. 1986; Weimer 1993) as well as the rumen (Clinquart et al. 1995; Kajikawa et al. 1997). Similar amounts of carbon dioxide (~ 30 mM) were formed with [^{12}C]-cellulose and [^{13}C]-cellulose treatments under oxic conditions (Figure 11A), indicating that both isotopic forms of cellulose were equally utilized. Carbon dioxide accumulated to approximately 2 mM (^{12}C) and 7 mM (^{13}C) under anoxic conditions, and concentrations of molecular hydrogen were below 0.3 mM in [^{12}C]-treatments, but about 7 mM in [^{13}C]-treatments (Figure 11A). Furthermore, the amounts of products and time at which they were produced varied between [^{12}C]- and [^{13}C]-cellulose treatments under anoxic conditions, but the pattern of product profiles was qualitatively similar (Figure 11A). The reasons for these discrepancies are unclear. Organic products were negligible during the first several weeks of incubation in anoxic treatments supplemented with cellulose, followed by the accumulation of propionate (~ 2 mM) and acetate (3 – 7 mM).

[^{12}C]- and [^{13}C]-cellobiose, and -glucose were pulsed periodically at low concentrations (~ 0.25 mM per pulse) so that the soil microbial community was not subjected to concentrations higher than necessary. Both cellobiose and glucose were consumed without apparent delay in oxic and anoxic treatments (Figure 11B and C), indicating that the microbial community was poised to aerobically and anaerobically consume these substrates. Approximately 3 mM cellobiose or glucose was degraded in total, corresponding to consumed carbon of 110 $\mu\text{mol g}_{\text{soilDW}}^{-1}$ and 55 $\mu\text{mol g}_{\text{soilDW}}^{-1}$, respectively. A transient accumulation of glucose in oxic cellobiose-supplemented treatments was observed, whereas glucose was not detected in cellobiose-supplemented treatments under anoxic conditions (Figure 11B). In contrast to cellulose-supplemented treatments, no major difference in the amount of degradation products between the [^{12}C]- and [^{13}C]-isotope in cellobiose- and glucose-supplemented treatments was observed, and product profiles were similar. Product profiles were also similar between cellobiose and glucose, though the amounts of soluble carbonaceous products were higher in cellobiose-supplemented treatments, a result consistent with the fact that cellobiose has two glucose equivalents. Concentrations of carbon dioxide increased up to 20 mM in cellobiose-supplemented treatments (Figure 11B) and up to 10 mM in glucose-supplemented treatments (Figure 11C). Acetate was the main soluble carbonaceous product under anoxic conditions and accumulated up to 8 mM, followed by butyrate (~ 3.5 mM) and propionate (~ 2 mM) (Figure 11B and C). Small amounts of isobutyrate (< 0.15 mM) were occasionally detected (data not shown). The production of molecular hydrogen

(~10 mM) was only detected in anoxic treatments and paralleled the production of carbon dioxide. Methane was not detected in any of the treatments. Carbon recoveries were between 46% and 56% in oxic cellobiose- and oxic glucose-supplemented treatments, and above 100% in anoxic treatments.

Nitrate was present at the beginning of incubation at concentrations approximating 0.6 ± 0.1 mM and was consumed in both oxic and anoxic treatments supplemented with cellulose, cellobiose, and glucose. The lack of detectable nitrate after 7 days in cellulose-supplemented treatments (oxic and anoxic), and 48 hours (anoxic) or 144 hours (oxic) in cellobiose- and glucose-supplemented treatments (data not shown) indicated that nitrate was either dissimilated or assimilated. Concentrations of ferrous iron increased in all anoxic treatments (Figure 11) after nitrate was consumed. Once formed, ferrous iron did not appear to be a stable end product in cellulose-supplemented treatments in which concentrations of nitrate decreased. pH was stable at approximately 6.5 ± 0.2 in oxic treatments but increased to approximately 7.4 ± 0.2 in anoxic treatments (data not shown).

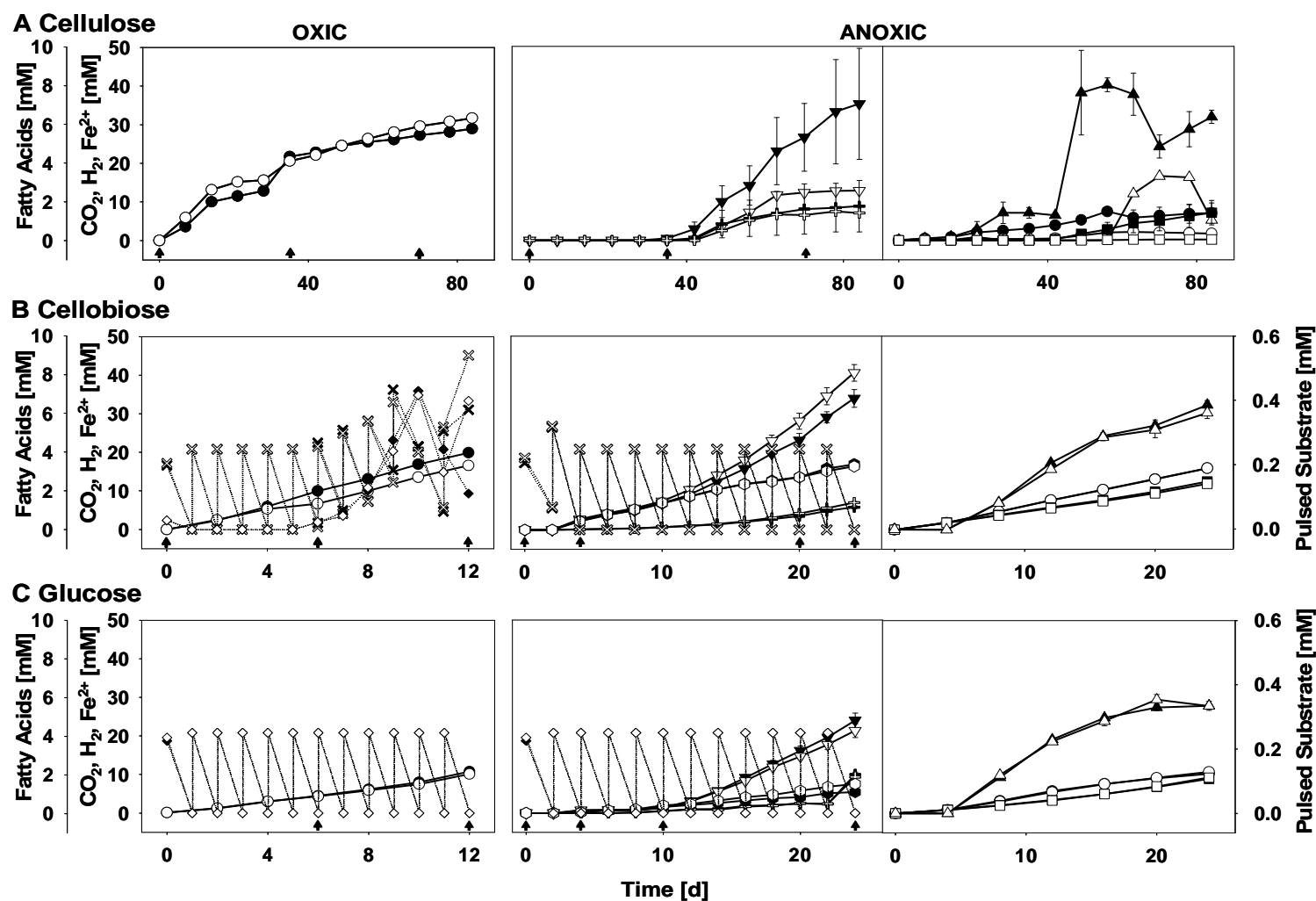


Figure 11. Degradation of cellulose (A), cellobiose (B), and glucose (C) in soil slurries. Values are the means of triplicates (¹³C-cellobiose, ¹³C-glucose), duplicates (¹³C-cellulose, ¹²C-cellobiose, ¹²C-glucose), or a single treatment (¹²C-cellulose). Error bars indicate standard deviations. Concentrations of compounds in unsupplemented treatments were subtracted from concentrations of products in supplemented treatments; the difference is shown. Solid symbols, values from treatments supplemented with ¹³C-substrates; Open symbols, values from treatments supplemented with ¹²C-substrates. Symbols: * cellobiose; ◆ glucose; ▼ acetate; ● butyrate; + propionate; • carbon dioxide; ■ molecular hydrogen; ▲ ferrous iron. Arrows indicate sampling for stable isotope probing analyses.

3.2. Identification of Active Prokaryotes in [¹³C]-Cellulose, -Cellobiose, and -Glucose Supplemented Treatments by RNA Stable Isotope Probing

Stable isotope probing was used to identify prokaryotic soil organisms that directly incorporated [¹³C]-carbon into their RNA by assimilating [¹³C]-cellulose, -cellobiose, or -glucose under oxic and anoxic conditions (2.3.1, 3.1). Although the risk of cross-feeding by [¹³C]-carbon dioxide was minimized (2.3.1) it cannot be excluded that some soil organisms incorporated [¹³C]-carbon by assimilation of [¹³C]-breakdown products (e.g., fatty acids) during prolonged incubation. Therefore, tRFLP analysis was not only performed at the end of incubation but also in between to detect time-dependent shifts in the active microbial community (Figure 11).

3.2.1. Distribution of RNA in Gradient Fractions and Selection of 'Light' and 'Heavy' Fractions for Molecular Analyses

RNA extracted from [¹²C]- and [¹³C]-cellulose, -cellobiose, or -glucose treatments was subjected to isopycnic centrifugation in a density gradient to separate 'heavy' [¹³C]-RNA of saccharide-assimilating, i.e., labeled, organisms from 'light' [¹²C]-RNA of non-labeled organisms. A RNA-free gradient (blank) was used to determine the buoyant density of any fraction (Figure 12).

The buoyant density of each fraction decreased linearly and gradients were reproducible and comparable between three independent centrifugation runs (Figure 12). Buoyant densities ranged from $1.84 \pm 0.01 \text{ g ml}^{-1}$ to $1.76 \pm 0.01 \text{ g ml}^{-1}$. Since the gradients were highly reproducible (Figure 12) the distribution of RNA in gradient fractions was only determined exemplarily for one gradient (Figure 13).

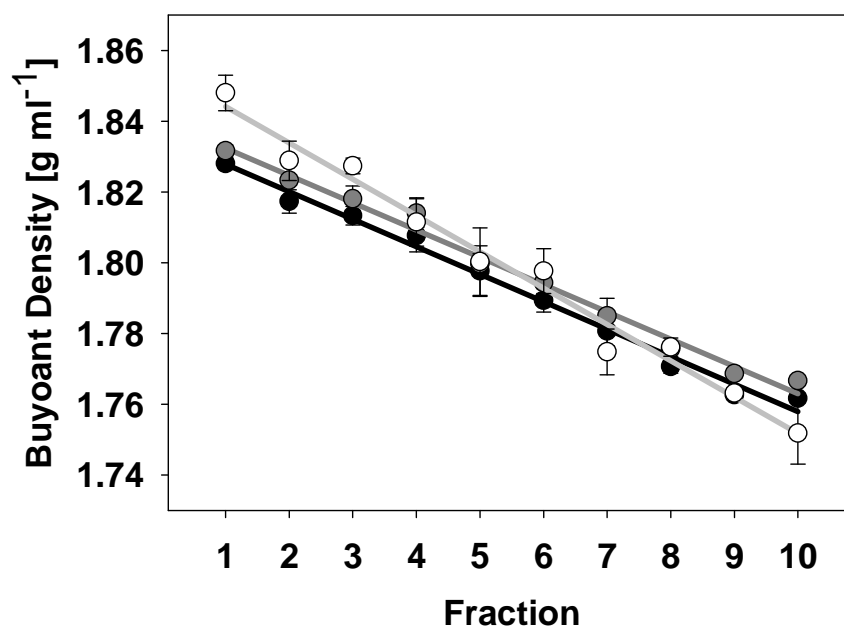


Figure 12. Buoyant density of gradient solution in fractions of blank gradients that lack RNA at 25°C. Closed symbols: centrifugation run 1; grey symbols: centrifugation run 2; open symbols: centrifugation run 3. Lines represent linear regressions ($R^2 = 0.98$). Error bars indicate standard deviations ($n = 10$).

RNA was distributed over the whole gradient with highest concentrations in fractions number four and seven, indicating a partial separation of [^{13}C]- and [^{12}C]-RNA (Figure 12). Full separation of ‘heavy’ and ‘light’ RNA derived from pure cultures is possible (Lüders et al. 2004), but not likely for environmental samples. Based on literature (Lüders et al. 2004; Manfield et al. 2002a) two fractions that presumably represented labeled ‘heavy’ or unlabeled ‘light’ RNA were selected for further analyses. Fractions number three and four (buoyant densities of $1.813 \pm 0.00 - 1.821 \pm 0.01 \text{ g ml}^{-1}$) were pooled and correspond to ‘heavy’ RNA. Fractions number eight and nine (buoyant densities of $1.767 \pm 0.00 - 1.776 \pm 0.00 \text{ g ml}^{-1}$) were pooled for analyses of unlabeled ‘light’ RNA (Figure 13). RNA of ‘heavy’ and ‘light’ fractions of [^{12}C]- and [^{13}C]-treatments was transcribed into cDNA and used for comparative tRFLP analyses of bacterial and archaeal 16S rRNA cDNA sequences.

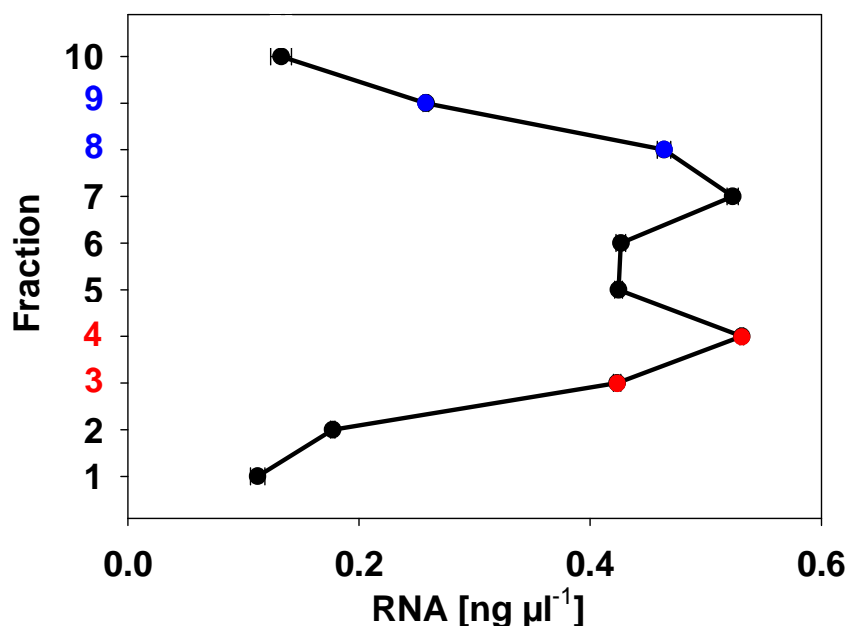


Figure 13. Distribution of RNA in gradient fractions of anoxic [¹³C]-cellobiose treatments after 24 days of incubation. Red indicates fractions that were chosen for analyses of ‘heavy’ RNA. Blue indicates fractions that were chosen for analyses of ‘light’ RNA. Error bars indicate the standard deviation (n = 3). Buoyant density of ‘light’ fractions was $1.767 \pm 0.00 - 1.776 \pm 0.00 \text{ g ml}^{-1}$. Buoyant density of ‘heavy’ fractions was $1.813 \pm 0.00 - 1.821 \pm 0.01 \text{ g ml}^{-1}$.

3.2.2. TRFLP Profiles of Archaeal 16S rRNA cDNA Sequences

Archaeal 16S rRNA cDNA genes were digested with *TaqI*, resulting in two major tRFs (142 bp, 154 bp) and some minor tRFs with relative fluorescence values below 5% (Figure 14). These tRFs were present in each sample of cellulose-, cellobiose-, or glucose-supplemented treatments (data not shown). Neither differences between [¹³C]- and [¹²C]-treatments nor between ‘light’ and ‘heavy’ fractions of [¹³C]-treatments were detected (Figure 14). This general result suggested that mesophilic *Archaea* were of minor to no consequence to the primary consumption of supplemental substrates. Archaeal 16S rRNA cDNA genes were not used for cloning, but *in silico* analyses revealed that the tRFs might represent species of *Methanocorpusculaceae*, *Methanococcaceae*, and *Methanobacteriaceae* (tRF 142 bp). TRF 154 bp could not be assigned to known sequences.

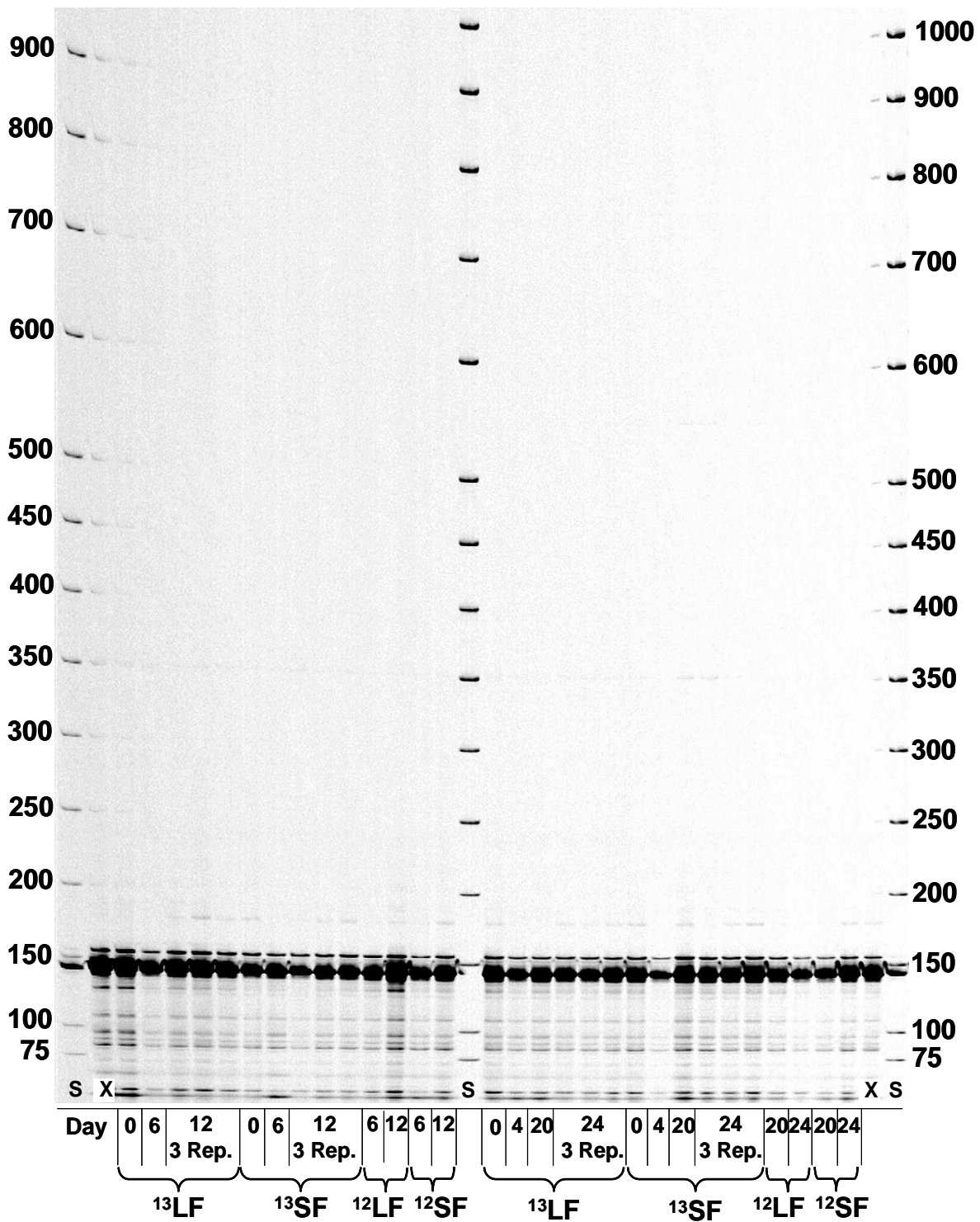


Figure 14. TRFLP gel image of archaeal 16S rRNA cDNA genes. Restriction enzyme was *TaqI*. S, length standard 75 – 1,000 bp; LF, 'light' fraction; SF, 'heavy' fraction; Rep., Replicates; X, Lane was not analysed.

3.2.3. TRFLP Profiles of Bacterial 16S rRNA cDNA Sequences

Primarily, tRF profiles analysed of the last time points were compared, i.e., for each treatment 'light' and 'heavy' fraction of oxic and anoxic treatments supplemented with either [^{13}C]-cellulose, [^{13}C]-cellobiose, [^{13}C]-glucose, [^{12}C]-cellulose, [^{12}C]-cellobiose, or [^{12}C]-glucose were compared (Figure 15 and Figure 16). Separation of [^{12}C]- and [^{13}C]-RNA during centrifugation should be reflected in different tRF patterns between 'light' and 'heavy' fractions.

3.2.3.1. Comparison of 'Heavy' and 'Light' Fractions in Oxic and Anoxic [^{12}C]- or [^{13}C]-Treatments

Restriction digestion of bacterial 16S rRNA cDNA sequences derived from [^{13}C]-treatments resulted in different tRF pattern of the 'light' and 'heavy' fractions (Figure 15), confirming incorporation of [^{13}C]-carbon into RNA by parts of the microbial community and successful separation of [^{12}C]- and [^{13}C]-RNA in the gradient solution. Assuming that labeled taxa are presented by (i) every tRF that is exclusively present in the 'heavy' fraction but not in the 'light' fraction and (ii) every tRF that exhibits a higher relative fluorescence in the 'heavy' than in the 'light' fraction, 31 tRFs (in total) were putatively labeled in [^{13}C]-treatments at the end of incubation.

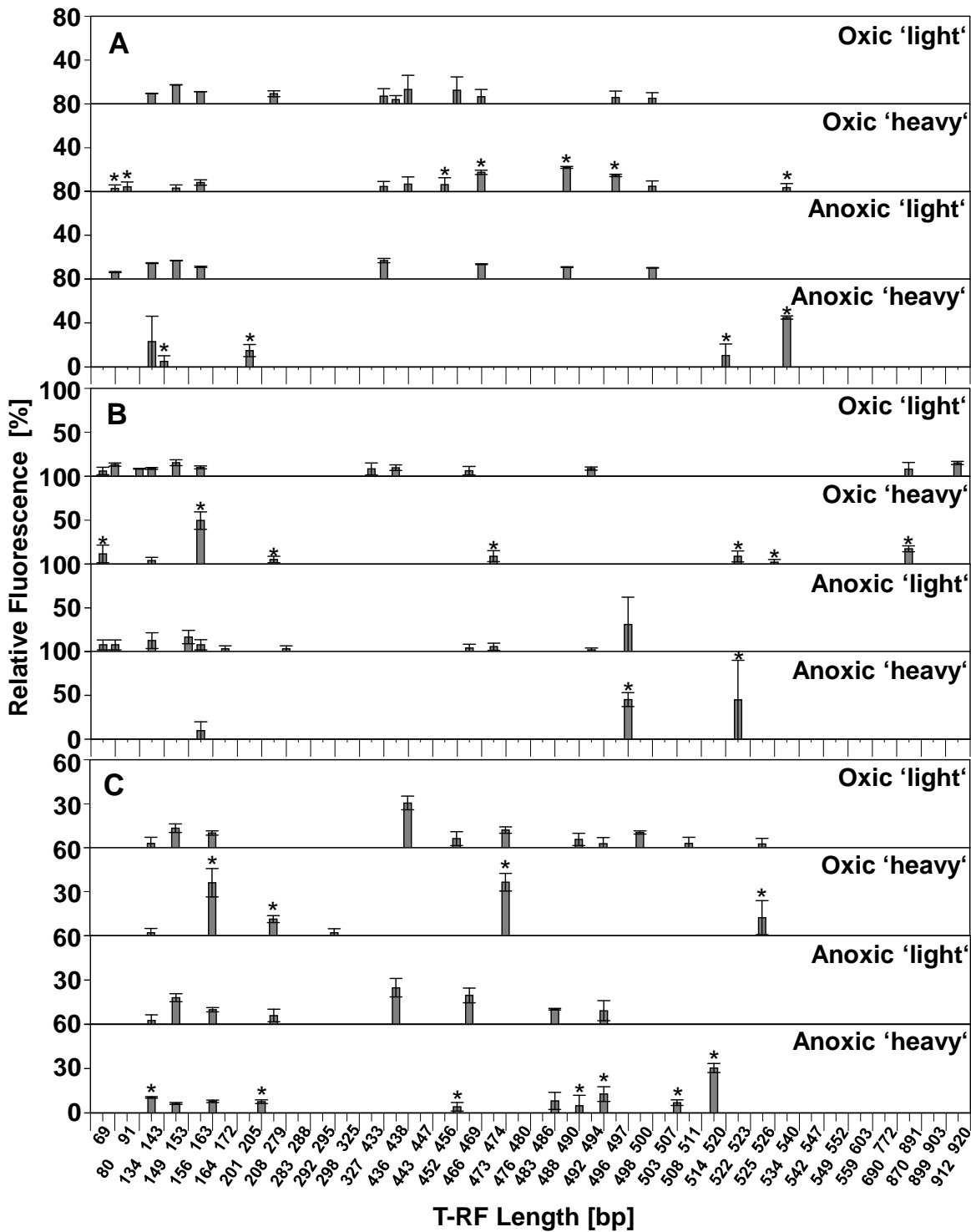


Figure 15. TRFLP pattern of bacterial 16S rRNA cDNA sequences in 'light' (fraction 8+9: $1.767\text{-}1.776\text{ g ml}^{-1}$) and 'heavy' fractions (fraction 3+4: $1.181\text{-}1.183\text{ g ml}^{-1}$) of $[^{13}\text{C}]$ -supplemented treatments at the latest analysed time point. (A) Cellulose treatment after 70 days. (B) Cellobiose treatment after 12 days (oxic) and 24 days (anoxic). (C) Glucose treatment after 12 days (oxic) and 24 days (anoxic). Values are the mean of triplicates ($[^{13}\text{C}]$ -cellobiose, $[^{13}\text{C}]$ -glucose) or duplicates ($[^{13}\text{C}]$ -cellulose). Error bars indicate standard deviations. Bars that represent putatively labeled tRFs (i.e., tRFs exclusively occurred in 'heavy' fractions or are higher in relative fluorescence than the corresponding tRFs in 'light' fractions) are marked with an asterisk.

Comparisons of 'light' and 'heavy' fractions may lead to an overestimation of the active part of the microbial community, since tRFs in the 'heavy' fraction do not necessarily result from labeled RNA, but can result from co-migrated unlabeled RNA (Lüders et al. 2004). The presence of unlabeled tRFs in 'heavy' fractions becomes apparent by comparison of 'heavy' and 'light' fractions of [^{12}C]-treatments (Figure 16) that never received [^{13}C]-carbon. In total, eight tRFs were exclusively present in 'heavy' fractions of [^{12}C]-treatments, although a [^{13}C]-labeling was impossible. These unlabeled genotypes in 'heavy' fractions could be related to sequences that had high G+C contents (53 – 61%, 3.2.4.2.2). They may have thus been unfolded RNA molecules yielding buoyant densities similar to labeled genotypes (Lüders et al. 2004). To avoid this bias identification of labeled tRFs was based on the comparison of 'heavy' fractions of [^{12}C]-treatments with 'heavy' fractions of [^{13}C]-treatments (3.2.3.2).

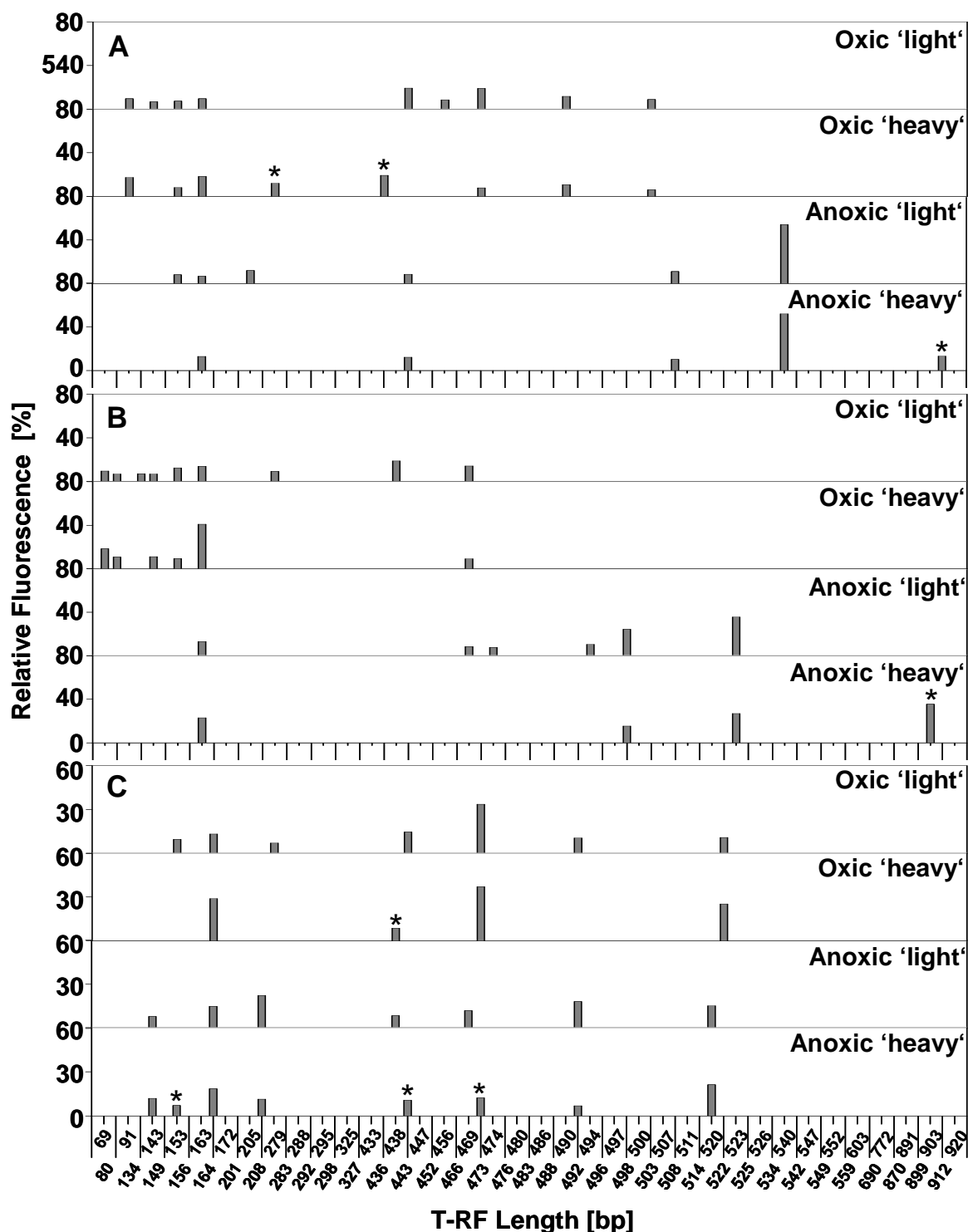


Figure 16. TRFLP pattern of bacterial 16S rRNA cDNA sequences in 'light' (fraction 8+9: 1.767-1.776 g ml⁻¹) and 'heavy' fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of [¹²C]-supplemented treatments at the latest analysed time point. (A) Cellulose treatment after 70 days. (B) Cellobiose treatment after 12 days (oxic) and 24 days (anoxic). (C) Glucose treatment after 12 days (oxic) and 24 days (anoxic). Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Bars that represent tRFs that exclusively occur in 'heavy' fractions are marked with an asterisk.

3.2.3.2. Identification of Labeled tRFs by Comparison of ‘Heavy’ Fractions between [¹²C]- and [¹³C]-Treatments and Phylogenetic Affiliation

A conservative approach was performed to identify labeled tRFs (3.2.3.1). TRFs were scored as labeled when they were only present in [¹³C]-profiles (e.g., tRF 507 bp, Figure 17) or when the relative intensity in [¹³C]-profiles was significantly higher than in [¹²C]-profiles at the same respective time interval (e.g., tRF 490 bp, Figure 17). Labeled tRFs were assigned to family-level taxa based on gene library analyses (Table 19 – Table 22).

3.2.3.2.1. *Bacteria that Incorporated [¹³C]-Carbon in Oxic Treatments*

[¹³C]-cellulose supplementation resulted in twelve labeled tRFs of which tRFs 438, 466, 490, 507, 525, and 540 bp were observed at days 35 and 70, whereas tRFs 80, 443, 456, 473, 497, and 503 bp were only detected at day 70 (Figure 17). No tRF was dominant or contributed to more than 30% to the total fluorescence what correlated with the frequency of corresponding labeled genotypes obtained from gene libraries from oxic cellulose-supplemented treatments (Table 22, 3.2.4.2.1). Ten tRFs were identified as known and new family-level taxa within the phyla *Actinobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Chloroflexi*, *Deltaproteobacteria*, *Firmicutes*, *Gammaproteobacteria*, *Planctomycetes*, and two deep-branching groups within *Bacteria* (‘Bac2’) and *Proteobacteria* (‘Prot1’) (Table 19). Two tRFs (503 bp, 540 bp) could not be identified.

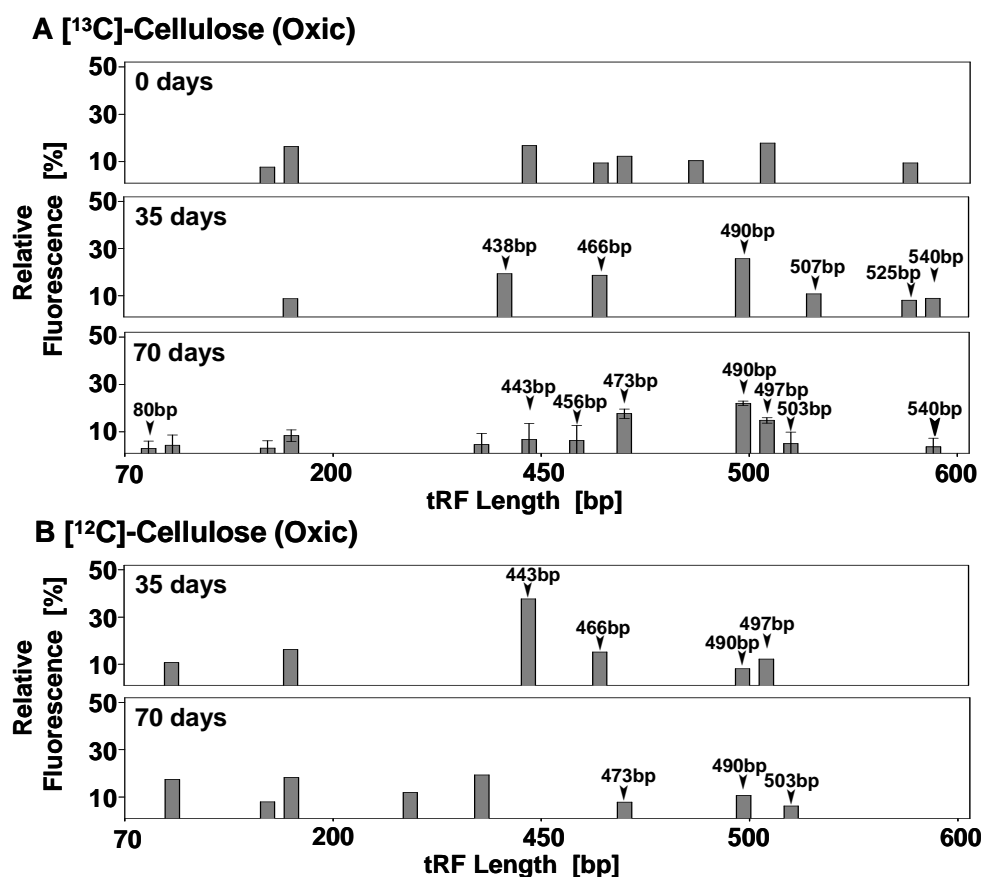


Figure 17. TRFLP pattern of bacterial 16S rRNA cDNA sequences in 'heavy' fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of cellulose-supplemented oxic treatments. (A) [¹³C]-cellulose treatment after 0, 35, and 70 days. (B) [¹²C]-cellulose treatment after 35 and 70 days. Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-labeled tRFs are indicated by their length and marked by an arrow (80 bp; 438 bp; 443 bp; 456 bp; 466 bp; 473 bp; 490 bp; 497 bp; 503 bp; 507 bp; 525 bp; 540 bp).

[¹³C]-cellobiose supplementation resulted in six labeled tRFs of which tRFs 163 and 279 bp increased in relative fluorescence most remarkably from day 0 to day 6 in [¹³C]-treatments, but decreased in [¹²C]-treatments. TRF 163 bp showed also a high fluorescence at day 12 (Figure 18). Both tRFs were affiliated with the phylum *Actinobacteria* (Table 19) which was a dominant taxon in clone libraries (Table 22). Additionally, four labeled tRFs (i.e., 474, 523, 534, and 890 bp) were detected at day 12 (Figure 18). Although one of these tRFs (i.e. tRF 890 bp) exhibited relative fluorescence of ~20%, none could be identified (Table 19).

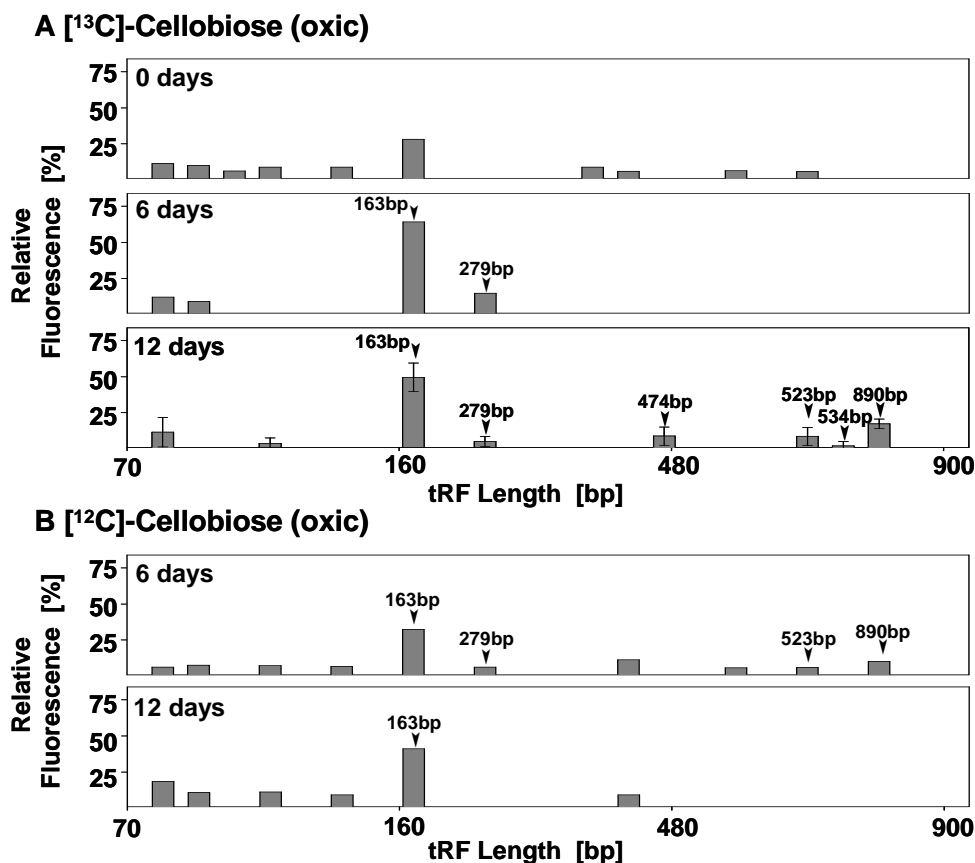


Figure 18. TRFLP pattern of bacterial 16S rRNA cDNA sequences in ‘heavy’ fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of cellobiose-supplemented oxic treatments. (A) [¹³C]-cellobiose treatment after 0, 6, and 12 days. (B) [¹²C]-cellobiose treatment after 6 and 12 days. Time points were chosen concerning changes in processes during substrate utilization (Figure 11). The [¹²C]-gradient for day 0 was not generated. Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-labeled tRFs are indicated by their length and marked by an arrow (163 bp; 279 bp; 474 bp; 523 bp; 534 bp; 890 bp).

[¹³C]-glucose supplementation resulted in six labeled tRFs of which tRFs 164, 476, and 526 bp occurred at day 6 (Figure 19). These tRFs were dominant and also observed at day 12. TRF 164 bp was present in ‘heavy’ fractions of [¹²C]-treatments, but had a higher relative fluorescence value in [¹³C]-treatments. Some minor tRFs (i.e., 143, 279, and 298 bp) appeared at day 12 (Figure 19), indicating a time-dependent shift of the active community. TRFs 164, 476, and 526 bp were affiliated with different families of the phyla *Actinobacteria* and *Betaproteobacteria* (Table 19) and were prevalent in gene libraries (Table 22). TRFs 143 and 279 bp affiliated with families of the phyla *Actinobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria* (Table 19). TRF 298 could not be identified.

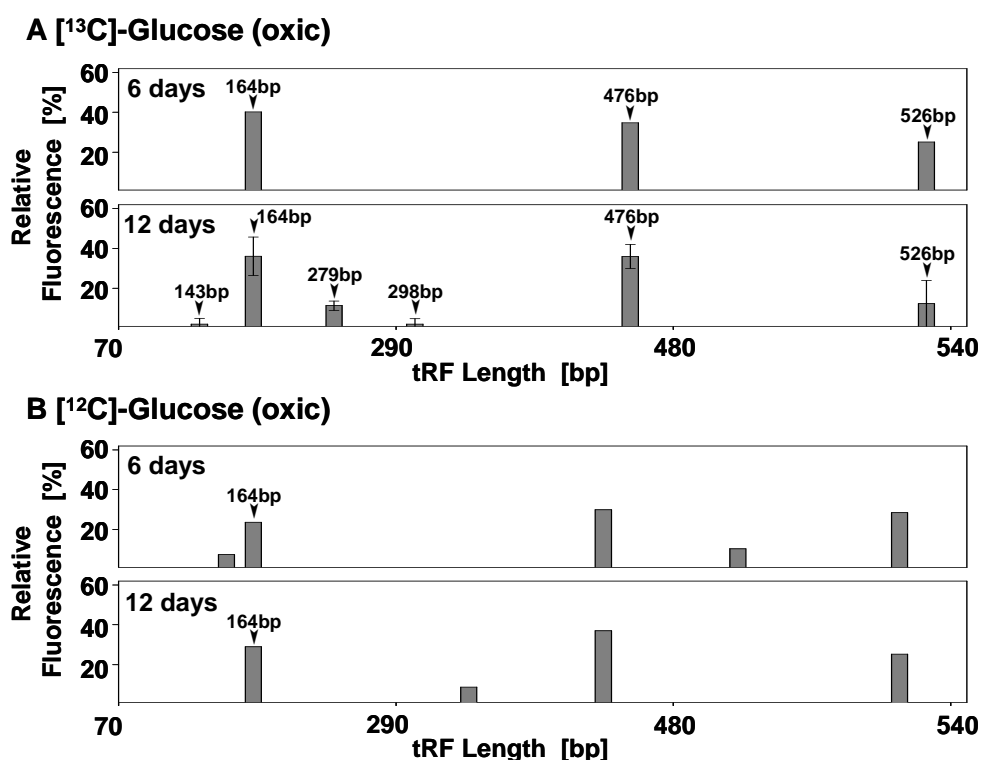


Figure 19. TRFLP pattern of bacterial 16S rRNA cDNA sequences in ‘heavy’ fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of glucose-supplemented, oxic treatments. (A) [¹³C]-glucose treatment after 6, and 12 days. (B) [¹²C]-glucose treatment after 6 and 12 days. Time points were chosen concerning changes in processes during substrate utilization (Figure 11). Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-labeled tRFs are indicated by their length and marked by an arrow (143 bp; 164 bp; 279 bp; 298 bp; 476 bp; 526 bp).

Cloning and *in silico* analyses of 16S rRNA gene sequences amplified from cDNA of ‘heavy’ fractions of oxic [¹³C]-cellulose, -cellobiose, and -glucose treatments were used to assign each labeled tRF (Figure 17 – Figure 19) to the bacterial family-level taxon it likely represents (Table 19, Figure A1 – Figure A3).

Table 19. Identification and occurrence of labeled tRFs in oxic [¹³C]-treatments.

Treatment	tRF [bp]	Identity [phylum: family] ^a	Label ^b [day]	
Cellulose-supplemented				
Oxic	80	<i>Actinobacteria: Intrasporangiaceae</i> , 'Actino1' ^c <i>Bacteroidetes: 'Sphingo1-4'^c <i>Planctomycetes: Planctomycetaceae</i></i>	70	
	438	<i>Alphaproteobacteria: 'Rhizo1'^c</i>	35	
	443	<i>Alphaproteobacteria: 'Rhizo2'^c</i>	70	
	456	<i>Firmicutes: 'Clos3'^c</i>	70	
	466	<i>Actinobacteria: 'Micro1'^c <i>Deltaproteobacteria: 'Desu1'^c</i></i>	35	
	473	<i>Actinobacteria: Nocardiodaceae</i>	70	
	490	<i>Betaproteobacteria: Oxalobacteraceae</i> <i>Gammaproteobacteria: 'Chrom1'^c</i>	35, 70	
	497	<i>Proteobacteria: 'Prot1'^c</i>	70	
	503	n.i.	70	
	507	<i>Actinobacteria: Cellulomonadaceae</i> <i>Chloroflexi: 'Deha1'^c <i>Deltaproteobacteria: 'Myxo1'^c</i></i>	35	
	525	<i>Actinobacteria: 'Micro4'^c <i>Bacteria: 'Bac2'^c <i>Chloroflexi: 'Deha1'^c</i></i></i>	35	
	540	n.i.	35, 70	
	Cellobiose-supplemented			
	Oxic	163	<i>Actinobacteria: Cellulomonadaceae, Intrasporangiaceae, Kineosporiaceae, Micrococcaceae, 'Micro2'^c, Nocardiaceae</i>	6, 12
		279	<i>Actinobacteria: Micrococcaceae, Mycobacteriaceae</i>	6, 12
		474	n.i.	12
523		n.i.	12	
534		n.i.	12	
890		n.i.	12	

Treatment	tRF [bp]	Identity [phylum: family] ^a	Label ^b [day]
Glucose-supplemented			
Oxic	143	<i>Actinobacteria: Intrasporangiaceae, Kineosporiaceae, Micrococcaceae</i> <i>Deltaproteobacteria: Geobacteraceae</i>	12
	164	<i>Actinobacteria: Intrasporangiaceae, Micrococcaceae, 'Micro3'^c</i>	6, 12
	279	<i>Actinobacteria: Micrococcaceae</i> <i>Betaproteobacteria: 'Burk1'^c</i>	12
	298	n.i.	12
	476	<i>Actinobacteria: Nocardioideaceae</i> <i>Betaproteobacteria: Nitrosomonadaceae</i>	6, 12
	526	<i>Actinobacteria: 'Micro3'^c</i>	6, 12

n.i., Not identified.

^a Based on experimental gene libraries.

^b First labeling at noted day of incubations in 'heavy' RNA fractions.

^c New family-level taxon based on 16S rRNA gene similarities < 87% to next cultivated species.

Comparison of tRF profiles and 16S rRNA cDNA gene libraries of oxic [¹³C]-treatments revealed that genotypes belonging to the family *Planctomycetaceae* (phylum *Planctomycetes*), and five new family-level OTUs in the orders *Sphingobacteriales* ('Sphingo1', 'Sphingo2', 'Sphingo3', 'Sphingo4'; phylum *Bacteroidetes*) and *Dehalococcoidetes* ('Deha1'; phylum *Chloroflexi*) were exclusively found in cellulose-supplemented treatments (Table 19 and Table 22). In contrast, members of the phylum *Actinobacteria* were primary assimilators of substrate-derived carbon in treatments with glucose and cellobiose. The family *Micrococcaceae* was prevalent in both glucose- and cellobiose-supplemented treatments, whereas the family *Intrasporangiaceae* was only prevalent in glucose-supplemented treatments. Members of these two families were only minimally labeled in soils supplemented with cellulose (Table 22).

3.2.3.2.2. Bacteria that Incorporated [¹³C]-Carbon in Anoxic Treatments

TRFLP profiles of anoxic treatments demonstrated nicely the dynamic of microbial communities during prolonged incubation, as indicated by shifts in tRF patterns from day 0 at the later time points (Figure 20 - Figure 22). [¹³C]-cellulose supplementation resulted in seven tRFs of which tRFs 205, 490, 522, 540, and 899 bp occurred at day 35. Two additional tRFs (i.e., tRFs 143 and 149 bp) were observed at day 70 (Figure 20). Labeling patterns were different between [¹²C]- and [¹³C]-treatments. TRF 540 was present at both time points, but exclusively labeled at day 35 due to its high relative fluorescence in [¹²C]-treatments at day 70 (Figure 20). In contrast, tRFs 149, 205, 522, and 899 bp lacked completely in [¹²C]-treatments

(Figure 20). The most intensive tRFs were affiliated with frequently detected genotypes of the phyla *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* (Table 20 and Table 22). TRFs of lower relative fluorescence were assigned to *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and deep-branching groups of *Bacteria* ('Bac1' and 'Bac3'; Table 20 and Table 22). TRF 899 bp could not be identified (Table 20).

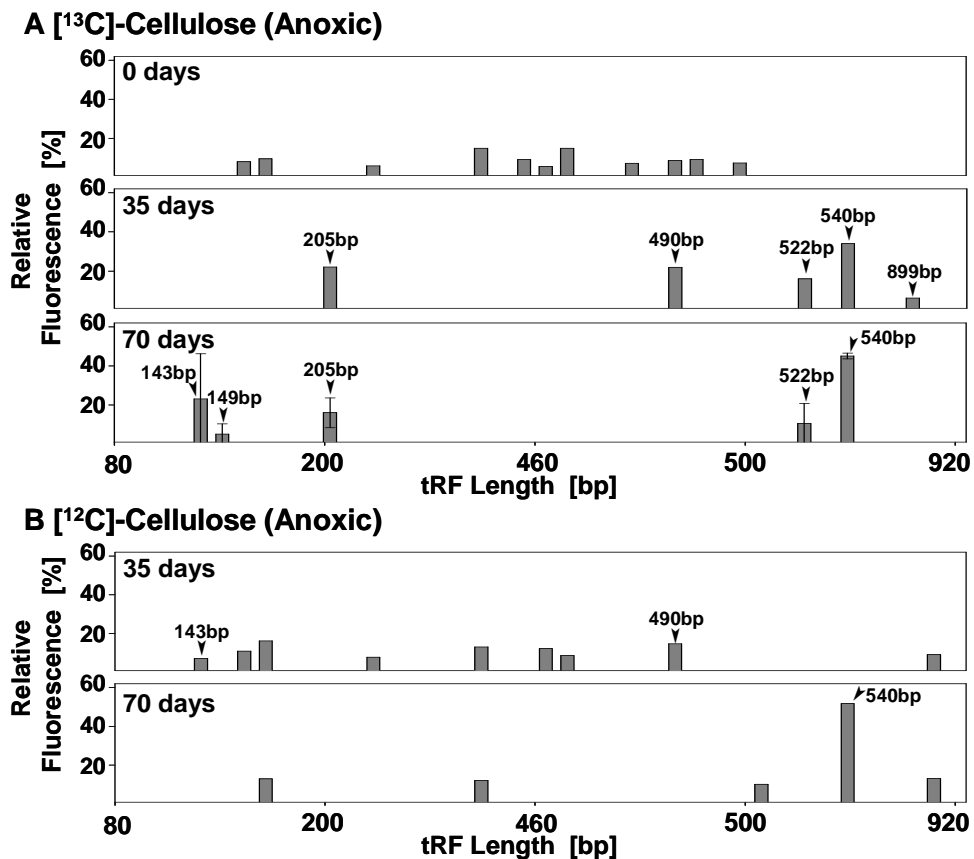


Figure 20. TRFLP pattern of bacterial 16S rRNA cDNA sequences in 'heavy' fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of cellulose-supplemented anoxic treatments. (A) [¹³C]-cellulose treatment after 0, 35, and 70 days. (B) [¹²C]-cellulose treatment after 0, 35, and 70 days. Time points were chosen concerning changes in processes during substrate utilization (Figure 11). The [¹²C]-gradient for day 0 was not generated. Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-labeled tRFs are indicated by their length and marked by an arrow (143 bp; 149 bp; 205 bp; 490 bp; 522 bp; 540 bp; 899 bp).

[¹³C]-cellobiose supplementation resulted in three labeled tRFs of which tRF 523 bp was present at day 4, 20, and 24, and exhibited the highest relative fluorescence (Figure 21). TRF 494 bp was only detected at day 4, tRF 903 at day 4 and 20, but disappeared at day 24 (Figure 21). The most dominant tRF 523 bp affiliated with the phylum *Firmicutes* (Table 20 and Table 22) what corresponds to the prevalence of this phylum in gene libraries (Table 22). TRF 494 bp represented members of the phyla *Gammaproteobacteria* and *Deltaproteobacteria*, taxa of minor importance in both relative fluorescence and frequencies in gene libraries (Table 20 and Table 22). TRF 903 bp could not be identified (Table 20).

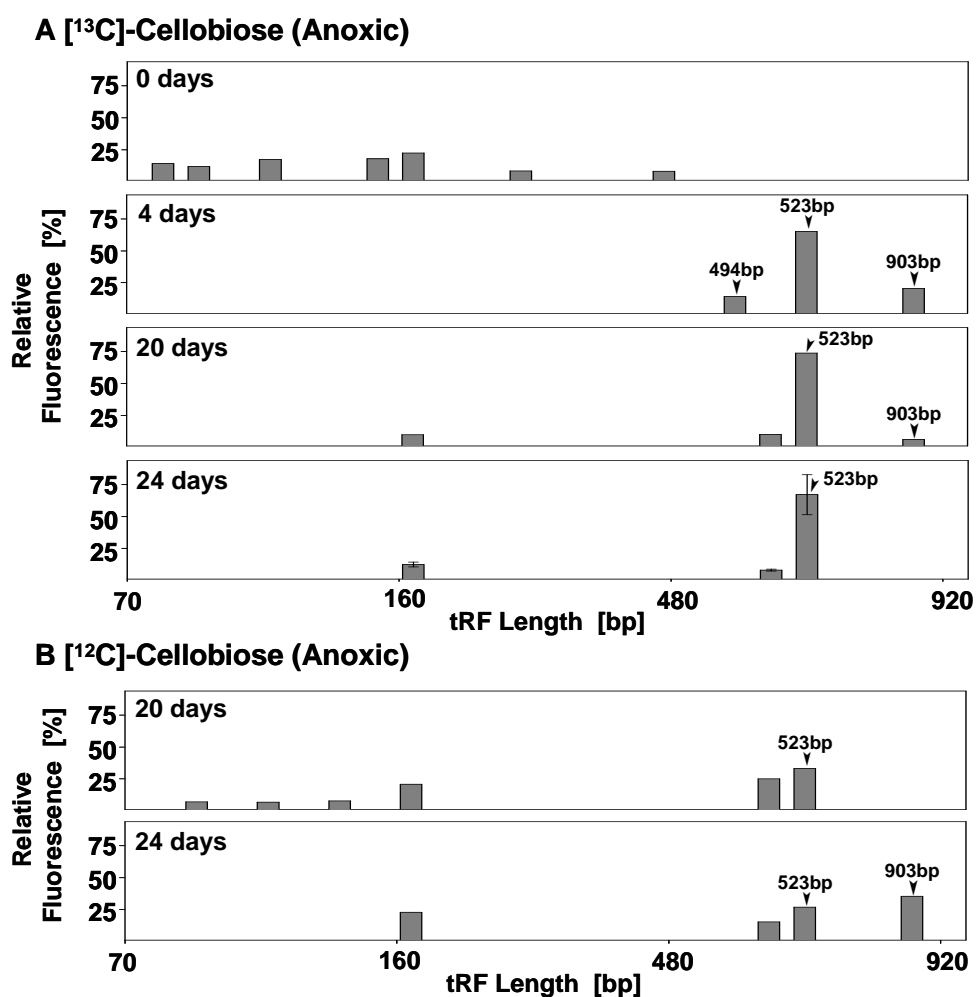


Figure 21. TRFLP pattern of bacterial 16S rRNA cDNA sequences in 'heavy' fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of cellobiose-supplemented anoxic treatments. (A) [¹³C]-cellobiose treatment after 0, 4, 10, and 24 days. (B) [¹²C]-cellobiose treatment after 20 and 24 days. Time points were chosen concerning changes in processes during substrate utilization (Figure 11). The [¹²C]-gradient for day 0 and 4 was not generated. Fluorescence values were normalized against the lowest single fluorescence; values below 5% were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-labeled tRFs are indicated by their length and marked by an arrow (494 bp; 523 bp; 903 bp).

[¹³C]-glucose supplementation resulted in three labeled tRFs (Figure 22) of which only tRF 520 bp was also present in [¹²C]-treatments. TRF 508 and 520 bp were only labeled at day 10 and 24, whereas tRF 496 was labeled at all time points (Figure 22). TRF 520 bp was the dominant labeled tRF and affiliated with the phylum *Firmicutes* which also represented the most frequent taxon in the corresponding gene library (Table 20 and Table 22). TRF 496 bp was assigned to the phylum *Gammaproteobacteria* (Table 20) which was a minor taxon in gene libraries (Table 22). TRF 508 bp could not be identified, although it exhibited a relative fluorescence of about 10%.

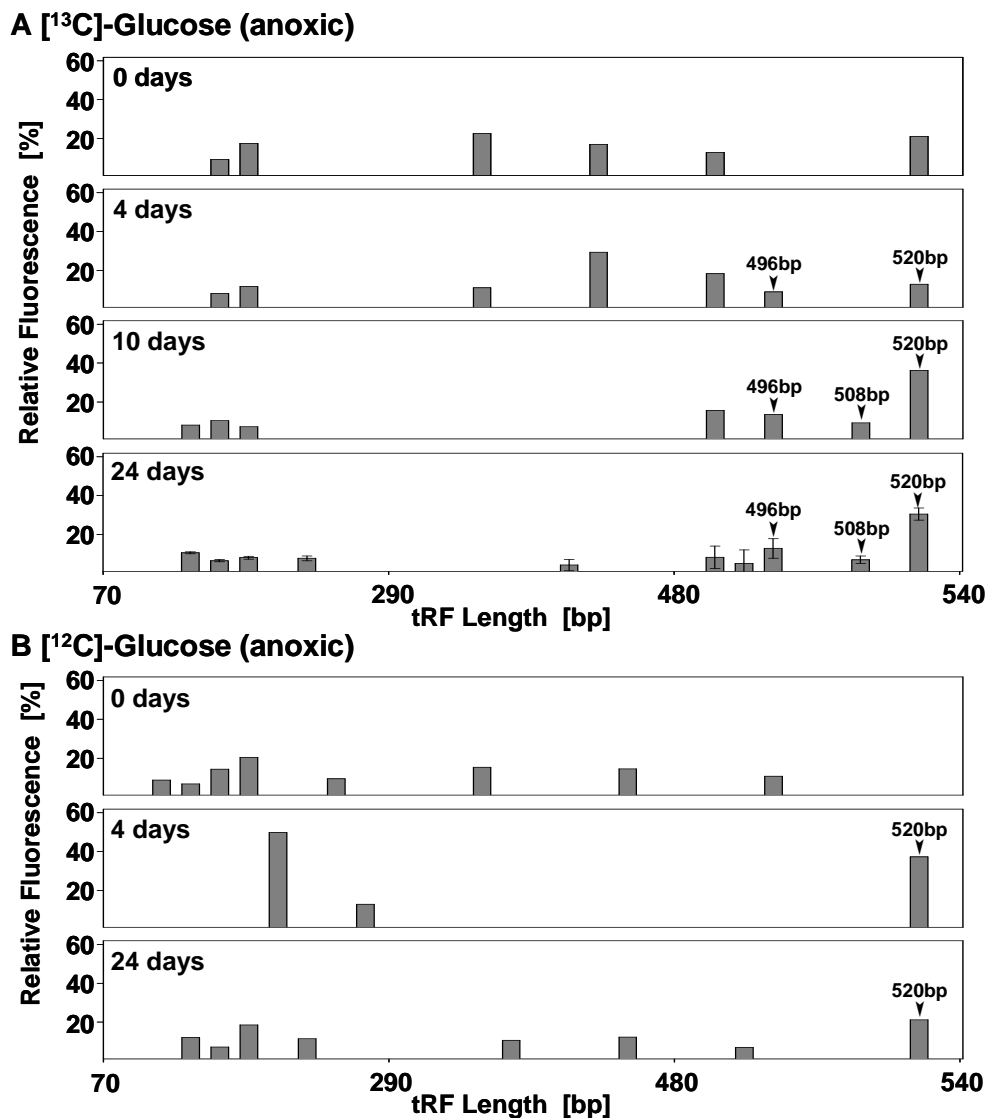


Figure 22. TRFLP pattern of bacterial 16S rRNA cDNA sequences in ‘heavy’ fractions (fraction 3+4: 1.181-1.183 g ml⁻¹) of glucose-supplemented anoxic treatments. (A) [¹³C]-glucose treatment after 0, 4, 10 and 24 days. (B) [¹²C]-glucose treatment after 0, 4, and 24 days. Time points were chosen concerning changes in processes during substrate utilization (Figure 11). The [¹²C]-gradient for day 10 was not generated. Fluorescence values were normalized against the lowest single fluorescence; values below 5 % were excluded from the analysis. Error bars indicate standard deviations. [¹³C]-Labeled tRFs are indicated by their length and marked by an arrow (496 bp; 508 bp; 520 bp).

TRFLP analyses and 16S rRNA cDNA gene libraries of anoxic [^{13}C]-treatments showed that the phylum *Firmicutes* was prevalent in all treatments, i.e., cellulose-, cellobiose-, and glucose-supplemented treatments (Table 22). Almost all labeled genotypes of the phylum *Firmicutes* were related to the family *Clostridiaceae*. Different sub-groups within this family were selectively activated in regard to the type of substrate. Sequences of Cluster III *Clostridiaceae* were closely related (> 97% sequence similarity) to the strict anaerobic cellulolytic species *Clostridium cellulolyticum* and *C. thermocellum* (Magnusson et al. 2009; Petitdemange et al. 1984; Zhang and Lynd 2005), whereas sequences derived from [^{13}C]-cellobiose and –glucose treatments were closely related (> 98% similarity) to the saccharolytic species *C. butyricum* and *C. vincentii* (Figure A2; Mountfort et al. 1997; Skerman et al. 1980). An unclassified cluster ('Cellu1') within the phylum *Bacteroidetes* and the family *Kineosporiaceae* (phylum *Actinobacteria*) were major active taxa in cellulose-supplemented treatments (Table 22, Figure A1 – Figure A3), but not in cellobiose- or glucose-supplemented treatments. The lack of these taxa in cellobiose and glucose-supplemented treatments (Table 22) indicates that labeling by cross-feeding on cellulose-derived cellodextrins was unlikely.

Table 20. Identification and occurrence of labeled tRFs in anoxic [^{13}C]-treatments.

Treatment	tRF [bp]	Identity [phylum: family] ^a	Label ^b [day]
Cellulose-supplemented			
Anoxic	143	<i>Actinobacteria: Intrasporangiaceae,</i> <i>Kineosporiaceae, Streptomycetaceae</i> <i>Deltaproteobacteria: Geobacteraceae</i>	70
	149	<i>Actinobacteria: Cellulomonadaceae</i> <i>Alphaproteobacteriaceae: Hyphomicrobiaceae</i>	70
	205	<i>Bacteroidetes: 'Cellu1'</i> ^c <i>Bacteria: 'Bac1'</i> ^c , ' <i>Bac3'</i> ^c <i>Firmicutes: Clostridiaceae, Paenibacillaceae,</i> <i>'Clos1'</i> ^c	35, 70
	490	<i>Betaproteobacteria: Rhodocyclaceae</i> <i>Deltaproteobacteria: Pelobacteraceae</i>	35
	522	<i>Firmicutes: Clostridiaceae, 'Clos2'</i> ^c	35, 70
	540	<i>Bacteroidetes: 'Cellu2'</i> ^c , ' <i>Cellu3'</i> ^c	35
	899	n.i.	35

Treatment	tRF [bp]	Identity [phylum: family] ^a	Label ^b [day]
Cellobiose-supplemented			
Anoxic	494	<i>Gammaproteobacteria: Aeromonadaceae</i> , 'Gam1' ^c	4
		<i>Deltaproteobacteria: Pelobacteraceae</i>	
	523	<i>Firmicutes: Clostridiaceae</i> , 'Clos4' ^c	4, 20, 24
	903	n.i.	4, 20
Glucose-supplemented			
Anoxic	496	<i>Gammaproteobacteria: Aeromonadaceae</i> , <i>Enterobacteriaceae</i>	4, 10, 24
	508	n.i.	10, 24
	520	<i>Firmicutes: Clostridiaceae</i>	10, 24

n.i., Not identified.

^a Based on experimental gene libraries.

^b First labeling at noted day of incubations in 'heavy' RNA fractions.

^c New family-level taxon based on 16S rRNA gene similarities < 87% to next cultivated species.

3.2.4. Bacterial 16S rRNA cDNA Gene Libraries of 'Heavy' Fractions

To identify tRFs related to labeled taxa, six 16S rRNA cDNA gene libraries were constructed, i.e., 16S rRNA cDNA genes derived from 'heavy' fractions of oxic and anoxic [¹³C]-cellulose, -cellobiose, or -glucose treatments were used for cloning.

3.2.4.1. Rarefaction and Coverage

In total, 828 sequences (> 350 bp length) were analysed. The number of clones per library ranged between 104 and 177 (Table 21). The coverage was above 90% in five of six libraries, indicating that the major diversity on family-level (> 87% sequence similarity) was covered by the analyses (Table 21). Some libraries exhibited a high number of sequences that could not be assigned to labeled tRFs. These sequences were defined to represent unlabeled bacterial taxa (Table 23), partly resulting in a low proportion of labeled clones in 'heavy' fractions (Table 21).

Table 21. Characteristics of 16S rRNA cDNA gene libraries of 'heavy' fractions of oxic and anoxic [^{13}C]-cellulose, [^{13}C]-cellobiose, and [^{13}C]-glucose treatments.

	Number of clones per library	Number of family-level OTUs	Labeled clones [%]	Unlabeled clones [%]	Coverage [%]
Cellulose-supplemented					
Oxic	163	93	23.3	76.7	64.4
Anoxic	177	27	89.8	10.2	93.8
Cellobiose-supplemented					
Oxic	122	19	45.2	54.8	93.4
Anoxic	130	17	64.6	35.4	94.7
Glucose-supplemented					
Oxic	104	15	63.6	36.4	96.2
Anoxic	132	28	23.3	76.7	90.9

Rarefaction analyses supported the above mentioned findings (i.e., detection of the major diversity), as indicated by the out-plateauing rarefaction curves (Figure 23). The rarefaction curve of oxic cellulose-supplemented treatments was steep (Figure 23 A) what corresponded to the low coverage of 64% of this gene library (Table 21). Gene libraries of oxic and anoxic cellobiose- and glucose-supplemented treatments showed both a good coverage (Table 21), and rarefaction curves nearly out-plateaued (Figure 23 B and C). In general, differences in the diversity within all treatments were observed. More family-level OTUs were detected in oxic cellulose- and oxic cellobiose-supplemented treatments than in the corresponding anoxic treatments, whereas oxic glucose-supplemented treatments showed less OTUs than the corresponding anoxic treatments (Table 21). The total number of detected OTUs was highest in cellulose-supplemented treatments.

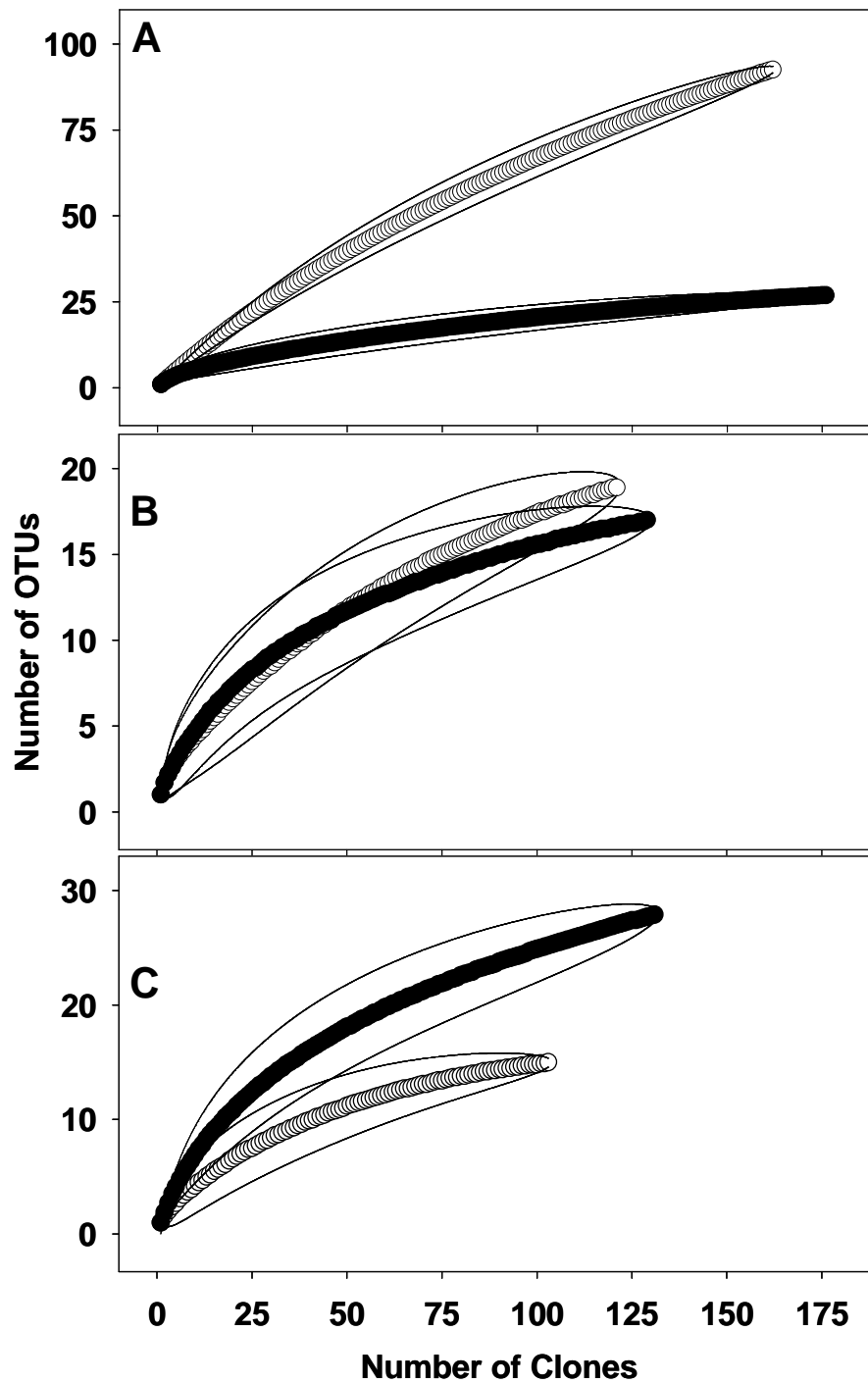


Figure 23. Rarefaction curves of 16S rRNA cDNA gene sequences of 'heavy' fractions of oxic and anoxic [^{13}C]-treatments. (A) Cellulose-supplemented; (B) Cellobiose-supplemented; (C) Glucose-supplemented. \circ , oxic treatments; \bullet , anoxic treatments. Solid lines indicate the 95% confidence intervals.

3.2.4.2. Grouping of Sequences into Family-level OTUs and their Phylogenetic Affiliation

16S rRNA cDNA gene sequences were grouped into family-level OTUs if sequence similarity was higher than 87 % (Yarza et al. 2008). TRFLP analysis was used to differentiate between genotypes of labeled and non-labeled taxa detected in 16S rRNA cDNA gene libraries.

3.2.4.2.1. *Labeled Taxa*

Forty-eight family-level taxa within ten bacterial phyla were identified as organisms that assimilated carbon derived from [¹³C]-cellulose, -cellobiose, or -glucose. Twenty-eight of these taxa did not closely affiliate with known families (Table 22, Figure A1 - Figure A3). Active taxa differed in regard to the substrate type and the availability of O₂, indicating that (i) cellulose supplementation selected for other taxa than cellobiose and glucose supplementation did, and (ii) oxic conditions selected for other taxa than anoxic conditions did (Table 22). Taxa that showed high relative abundances in gene libraries of oxic glucose- and cellobiose-supplemented treatments were the families *Intrasporangiaceae* (5 – 17%) and *Micrococcaceae* (30%) of the phylum *Actinobacteria* (Table 22, Figure A1). In contrast, major active taxa in anoxic glucose- and cellobiose-supplemented treatments were closely related to saccharolytic *Clostridiaceae* (Cluster I *Clostridiaceae*) of the phylum *Firmicutes* (19 – 53%) (Table 22, Figure A2). *Planctomycetaceae* (*Planctomycetes*), a deep-branching new family-level taxon ('Deha1') of the phylum *Chloroflexi*, and four new taxa ('Sphingo1-4') of *Sphingobacteriales* (*Bacteroidetes*) were the most abundant groups in gene libraries of oxic cellulose-supplemented treatments and exhibited relative abundances of almost 4%, 5%, and 3%, respectively (Table 22, Figure A3). *Kineosporiaceae* (*Actinobacteria*), cellulolytic *Clostridiaceae* (Cluster III *Clostridiaceae*, phylum *Firmicutes*), and three new deep-branching family-level taxa ('Cellu1-3') of the phylum *Bacteroidetes* were dominant in gene libraries of anoxic cellulose-supplemented treatments and had relative abundances of about 16%, 28%, and 22%, respectively (Table 22, Figure A1 – Figure A3). These groups were analysed for their response to changing availabilities of O₂ and pesticides in further experiments (3.6; 3.5).

Table 22. Relative abundances of labeled OTUs obtained from 16S rRNA cDNA gene libraries from ‘heavy’ fractions of [¹³C]-treatments and their phylogenetic affiliation.

Phyla and families	OTUs ^a	Relative Abundances [%]					
		Cellulose		Cellobiose		Glucose	
		oxic	anoxic	oxic	anoxic	oxic	anoxic
<i>Actinobacteria</i>							
<i>Cellulomonadaceae</i>	22, 23, 78	1.23	0.57	0.81	-	-	-
<i>Intrasporangiaceae</i>	5, 60, 79	0.61	1.14	4.84	-	16.8	-
<i>Kineosporiaceae</i>	2, 46	-	21.7	4.03	-	5.61	-
<i>Micrococcaceae</i>	3, 18, 42, 54, 57, 58, 59, 61, 80	-	-	29.8	-	29.9	-
<i>Mycobacteriaceae</i>	56, 62	-	-	2.24	-	-	-
<i>Nakamurellaceae</i>	55	-	-	-	-	0.93	-
<i>Nocardiaceae</i>	44, 65	-	-	1.61	-	-	-
<i>Nocardiodaceae</i>	13, 47, 63	0.61	-	-	-	4.67	-
<i>Streptomycetaceae</i>	11	-	2.29	-	-	-	-
‘Micro1’ (<i>Micrococcineae</i>) ^b	34	0.61	-	-	-	-	-
‘Micro2’ (<i>Micrococcineae</i>) ^b	41	-	-	0.81	-	-	-
‘Micro3’ (<i>Micrococcineae</i>) ^b	43	-	-	-	-	0.93	-
‘Micro4’ (<i>Micrococcineae</i>) ^b	48	0.61	-	-	-	-	-
‘Actino1’ (<i>Actinomycetales</i>) ^b	40	0.61	-	-	-	-	-
<i>Alphaproteobacteria</i>							
<i>Hyphomicrobiaceae</i>	72	-	0.57	-	-	-	-
‘Rhizo1’ (<i>Rhizobiales</i>) ^b	75	0.61	-	-	-	-	-
‘Rhizo2’ (<i>Rhizobiales</i>) ^b	76	0.61	-	-	-	-	-

Phyla and families	OTUs ^a	Relative Abundances [%]					
		Cellulose		Cellobiose		Glucose	
		oxic	anoxic	oxic	anoxic	oxic	anoxic
<i>Bacteroidetes</i>							
'Sphingo1' (<i>Sphingobacteriales</i>) ^b	28	1.23	-	-	-	-	-
'Sphingo2' (<i>Sphingobacteriales</i>) ^b	36	0.61	-	-	-	-	-
'Sphingo3' (<i>Sphingobacteriales</i>) ^b	38	0.61	-	-	-	-	-
'Sphingo4' (<i>Sphingobacteriales</i>) ^b	50	0.61	-	-	-	-	-
'Cellu1' ^c	6	-	13.7	-	-	-	-
'Cellu2' ^c	14	-	1.71	-	-	-	-
'Cellu3' ^c	74	-	0.57	-	-	-	-
<i>Betaproteobacteria</i>							
<i>Nitrosomonadaceae</i>	67	-	-	-	-	0.93	-
<i>Oxalobacteraceae</i>	66	0.61	-	-	-	-	-
<i>Rhodocyclaceae</i>	7	-	4.00	-	-	-	0.75
'Burk1' (<i>Burkholderiales</i>) ^b	24	-	-	-	-	0.93	-
<i>Chloroflexi</i>							
'Deha1' (<i>Dehalococcoidetes</i>) ^b	8	4.91	-	-	-	-	-
<i>Deltaproteobacteria</i>							
<i>Geobacteraceae</i>	27, 35	-	0.57	-	-	0.93	-
<i>Pelobacteraceae</i>	12	-	1.14	-	0.77	-	-
'Desu1' (<i>Desulfuromonadales</i>) ^b	19	1.23	-	-	-	-	-
'Myxo1' (<i>Myxococcales</i>) ^b	31	0.61	-	-	-	-	-

Phyla and families	OTUs ^a	Relative Abundances [%]					
		Cellulose		Cellobiose		Glucose	
		oxic	anoxic	oxic	anoxic	oxic	anoxic
<i>Firmicutes</i>							
<i>Clostridiaceae</i>	1, 4, 16, 20, 21, 25, 51, 52, 53, 68	-	28.0	-	53.1	-	18.8
<i>Paenibacillaceae</i>	15	-	0.57	-	-	-	-
'Clos1' (<i>Clostridiales</i>) ^b	29	-	1.14	-	-	-	-
'Clos2' (<i>Clostridiales</i>) ^b	37	-	0.57	-	-	-	-
'Clos3' (<i>Clostridiales</i>) ^b	49	0.61	-	-	-	-	-
'Clos4' (<i>Clostridiales</i>) ^b	82	-	-	-	0.77	-	-
<i>Gammaproteobacteria</i>							
<i>Aeromonadaceae</i>	70, 73	-	-	-	0.77	-	2.26
<i>Enterobacteriaceae</i>	9	-	-	-	-	-	1.50
'Chrom1' (<i>Chromatiales</i>) ^b	26	0.61	-	-	-	-	-
'Gam1' ^e	81	-	-	-	0.77	-	-
<i>Planctomycetes</i>							
<i>Planctomycetaceae</i>	10, 32, 33	3.68	-	-	-	-	-
<i>Proteobacteria</i>							
'Prot1' ^f	77	0.61	-	-	-	-	-
<i>Bacteria</i>							
'Bac1' ^g	17	-	1.71	-	-	-	-
'Bac2' ^h	30	0.61	-	-	-	-	-
'Bac3' ⁱ	64	-	1.14	-	-	-	-

-, Not detected.

^a Identification of OTUs by BLASTn (<http://blast.ncbi.nlm.nih.gov/>).

^b New family-level taxon based on 16S rRNA gene similarities < 87% to next cultivated species (Yarza et al. 2008).

^c Next cultivated species: *Prolixibacter bellaniivorans* (AY918928; 86% 16S rRNA gene similarity).

^d Sequences derived from cellulose-supplemented treatments clustered to Cluster III *Clostridiaceae*, sequences from cellobiose- and glucose-supplemented treatments clustered to Cluster I *Clostridiaceae* (according to Collins et al. 1994).

^e Next cultivated species: *Methylostrum kenyense* (DQ789390; 85% 16S rRNA gene similarity).

^f Next cultivated species: *Syntrophus acidiphilus* (U86447; 83% 16S rRNA gene similarity).

^g Next cultivated species: *Clostridium cellulolyticum* (X71847; 86% 16S rRNA gene similarity).

^h Next cultivated species: *Levilinea saccharolytica* (AB109439; 84% 16S rRNA gene similarity).

ⁱ Next cultivated species: *Clostridium cellulolyticum* (X71847; 84% 16S rRNA gene similarity).

3.2.4.2.2. *Non-labeled Taxa*

Thirty-five family-level taxa (i.e., 32 valid and 3 non-validated/new taxa) of eleven phyla were detected in gene libraries of 'heavy' fractions of [¹³C]-treatments (Table 23), but none of these taxa could be affiliated with labeled tRFs in tRFLP analyses. 337 sequences represented non-labeled families what corresponds to 44% of non-labeled sequences over all gene libraries. The relative abundance of non-labeled taxa over all sequences ranged from 0.1 to 21.1 (Table 23). The majority of sequences of non-labeled taxa had G+C contents between 56 – 61%, what might permit the co-migration of unlabeled RNA towards the 'heavy' fraction. Nevertheless, the lack of a labeling of these taxa in tRFLP profiles suggests, that they were likely of minor importance for the degradation of cellulose-derived carbon under the experimental conditions.

Table 23. Non-labeled family-level taxa obtained from 16S rRNA cDNA gene libraries from 'heavy' fractions of [¹³C]-treatments, their relative abundance over all 16S rRNA cDNA sequences, and cellulolytic isolates.

Phylum (Relative abundance [%])	Family	Representative Cellulolytic Isolate	Reference
<i>Acidobacteria</i> (1.1)	<i>Acidobacteriaceae</i>	<i>Telmatobacter bravus</i>	Pankratov et al. 2011
<i>Actinobacteria</i> (21.1)	<i>Acidimicrobiaceae</i>	-	-
	<i>Coriobacteraceae</i>	-	-
	<i>Microbacteriaceae</i>	-	-
	<i>Micromonosporaceae</i>	<i>Micromonospora chalcea</i>	Gallagher et al. 1996
	<i>Rubrobacteraceae</i>	-	-
	<i>Sanguibacteraceae</i>	-	-
	<i>Solirubrobacteraceae</i>	-	-
	<i>Sporichthyaceae</i>	-	-
<i>Alphaproteobacteria</i> (3.9)	<i>Beijerinckiaceae</i>	-	-
	<i>Bradyrhizobiaceae</i>	-	-
	<i>Caulobacteraceae</i>	-	-
	<i>Rhizobiaceae</i>	-	-
	<i>Rhodospirillaceae</i>	-	-
	<i>Sphingomonadaceae</i>	<i>Sphingomonas echinoides</i>	Wenzel et al. 2002
<i>Bacteroidetes</i> (1.4)	<i>Chitinophagaceae</i>	-	-
	<i>Cytophagaceae</i>	<i>Cytophaga hutchinsonii</i>	Nakagawa and Yamasato 1996
	<i>Flavobacteriaceae</i>	<i>Cellulophaga baltica</i>	Johansen et al. 1999
	<i>Sphingobacteriaceae</i>	<i>Mucilaginibacter dorajii</i>	Kim et al. 2010
<i>Betaproteobacteria</i> (5.2)	<i>Alcaligenaceae</i>	-	-
	<i>Comamonadaceae</i>	-	-
	<i>Methylophilaceae</i>	-	-
<i>Chloroflexi</i> (0.6)	<i>Anaerolineaceae</i>	-	-
	uncultured <i>Thermomicrobia</i>	-	-

Phylum (Relative abundance [%])	Family	Representative Cellulolytic Isolate	Reference
Deltaproteobacteria (6.0)	<i>Bacteriovoraceae</i>	-	-
	<i>Cystobacteraceae</i>	-	-
	<i>Desulfuromonadaceae</i>	-	-
	<i>Haliangiaceae</i>	-	-
	<i>Nannocystaceae</i>	-	-
	<i>Polyangiaceae</i>	<i>Sorganium cellulosum</i>	Lampky 1971
Firmicutes (1.9)	<i>Acidaminococcaceae</i>	-	-
	<i>Bacillaceae</i>	<i>Bacillus circulans</i>	Kim 1995
Fusobacteria (0.1)	<i>uncultured Fusobacteriales</i>	-	-
Gammaproteobacteria (2.0)	<i>Pseudomonodaceae</i>	<i>Cellvibrio mixtus</i>	Blackhall et al. 1985
Verrucomicrobia (0.5)	<i>Xiphinemato-bacteriaceae^a</i>	-	-

^a Family without validated nomenclature (<http://www.bacterio.cict.fr/>; last update 04.11.2011); Hedlund et al. 1997.

3.3. Labeled *Eukaryotes*

Some eukaryotic organisms were labeled in oxic cellulose-supplemented soil slurries (Table 24). No labeled *Eukaryotes* were detected under anoxic conditions. Ten tRFs represented labeled organisms. Three tRFs (i.e., 57, 378, and 382 bp) could not be identified by comparison with 18S rRNA cDNA gene libraries. Labeled species belonged to the families *Bodonidae*, *Eustigmataceae*, *Mallomonadaceae*, *Opisthnectidae*, unclassified *Chrysophyceae*, and unclassified *Stramenopiles*. Species related to *Ophisthnecta minima* and to a *Spumella*-like flagellate were the only organisms that were labeled at both investigated time points (Table 24). Although 18S rRNA cDNA sequences of fungi were detected in gene libraries (data not shown), no fungal taxon was labeled during the incubation. Further analyses are still in progress.

Table 24. Identification and occurrence of labeled eukaryotic tRFs in [¹³C]-cellulose treatments

tRF [bp]	Identity [family] ^a	Next related species (18S rRNA similarity) ^a	Label [day] ^b
Oxic			
57	n.i.	---	35
205	<i>Eustigmataceae</i>	<i>Vischeria punctata</i> (99%)	35
343	<i>Opisthnectidae</i>	<i>Opisthnecta minima</i> (99%)	35, 70
378	n.i.	---	35
382	n.i.	---	35
423	<i>Bodonidae</i>	<i>Neobodo designis</i> (99%)	35
430	Unclassified <i>Chrysophyceae</i> ^c	<i>Chrysophyceae</i> sp. CCCM41 (99%)	70
	<i>Mallomonadaceae</i>	<i>Synura curtispina</i> (94%)	
	<i>Mallomonadaceae</i>	<i>Mallomonas peroneides</i> (94%)	
452	<i>Bodonidae</i>	<i>Dimastigella mimosa</i> (98%)	70
454	Unclassified <i>Chrysophyceae</i> ^c	<i>Spumella</i> -like flagellate 1305 (98%)	35, 70
	Unclassified <i>Stramenopiles</i> ^d	<i>Leukarachnion</i> sp. ATCC PRA24 (92%)	
459	Unclassified <i>Chrysophyceae</i> ^c	<i>Spumella</i> -like flagellate 1305 (98%)	35, 70
	Unclassified <i>Stramenopiles</i> ^d	<i>Leukarachnion</i> sp. ATCC PRA24 (92%)	
Anoxic			
No Label.			

n.i., Not identified.

^a Identification by BLAST (<http://blast.ncbi.nlm.nih.gov/>).

^b First doubtless detection at day x in 'heavy' RNA fraction.

^c Taxonomic rank: 'class'.

^d No taxonomic rank of 'family' defined.

3.4. Design of Family-level qPCR Assays

Primers for quantification of family-level taxa (Table 15) were developed on the basis of 16S rRNA cDNA sequences gained in this study (2.5.12) and on a 16S rRNA gene database (Release 104) downloaded at <http://www.arb-silva.de> (Prüsse et al. 2007). ARB (Version 2005; <http://www.arb-home.de>, Ludwig et al. 2004) and the included software tools 'Probedesign' and 'Probematch' were used for construction of putative primers. The online tool 'Probematch' available at the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>; Cole et al. 2007; Cole et al. 2009) was additionally applied to check for primers with minimal binding to sequences of non-target organisms, i.e., organisms that do not cluster inside the corresponding family-level taxon (2.5.14). In total, 18 family-level primers complied with the above mentioned requirements, and were tested with DNA of target and non-target taxa.

At first, target DNA, i.e., 16S rRNA cDNA vector insert sequences of clones of the appropriate family-level taxon, was used to test if the primer pairs yielded a PCR product. Subsequently, the annealing temperature of every primer pair was determined on the basis of target and non-target DNA (i.e., clone insert or 16S rRNA sequences of organisms with exact two mismatches to the primer sequence; Table 15; Table 25). A TGradient Thermocycler (Biometra, Göttingen, Germany) was used for amplification of family-level 16S rRNA cDNA genes in target and non-target DNA in a temperature gradient (50 – 70°C). PCR products were checked by agarose gel electrophoreses (2.5.5). The temperature, at which the target DNA, but not the non-target DNA yielded a PCR product, was selected as approximated annealing temperature. The exact temperature for stringent annealing (Table 25) was optimized in closer gradients on an iQ5 iCycler (Bio-Rad, Hercules, USA). Not every primer pair yielded specific PCR products for target DNA, but eight tested primer pairs were presumably for application to environmental samples.

Table 25. Experimentally established parameters for specific qPCR measurements of family-level assays.

Assay no.	Primer pair ^a (FP / RP)	Target taxon	Annealing temp. (°C)	Product length (bp)	Target seq. (accession no.)	Non-target seq. (accession no.)
1	Gm5F / Micro1R	<i>Micrococcaceae</i> & <i>Cellulomonadaceae</i>	66.4	270	oxB151 (FN433954)	anoxB58 (FN433940)
2	Gm5F / Kin3R	<i>Kineosporiaceae</i> & <i>Nocardioideaceae</i>	67.5	290	anoxC150 (FN434006)	oxG32 (FN433963)
3	Gm5F / CloSac1R	Cluster I <i>Clostridiaceae</i>	64.0	550	anoxB228 (FN433944)	anoxB112 (FN433943)
4	Gm5F / CloCel2R	Cluster III <i>Clostridiaceae</i>	66.0	330	anoxC5 (FN433987)	anox118 (FN433934)
5	Pla4F / PlaGm5R	<i>Planctomycetaceae</i>	67.0	250	oxC15 (FN434018)	oxC115 (FR773529)
6	Gm5F / Cel4R	'Cellu1-3'	56.0	160	anoxC102 (FN433999)	oxC101 (FR773528)
7	Gm5F / Sph1R	'Sphingo1-4'	63.4	350	oxC47 (FN434021)	<i>S. myxococcoides</i> (AJ310654)
8	Gm5F / Deh1R	'Deha1'	66.3	260	oxC11 (FN434016)	anoxG57 (FR773527)

^a Details about primers in Table 15.

FP, Forward primer.

RP, Reverse primer.

no., Number.

seq., Sequence.

DNA from pure cultures was used for the design and optimization of family-level qPCR assays (2.5.10.1). Therefore, qPCR assays were checked for specific amplification of target sequences from soil derived cDNA (Table 26).

The *Micrococcaceae/Cellulomonadaceae* specific assay was first designed to target exclusively species of the family *Micrococcaceae* which was the dominant taxon in cellobiose- and glucose-supplemented treatments (3.2.4.2.1). *In silico* analyses showed that all putative primers additionally bind to sequences of the close related family *Cellulomonadaceae*. This family was also part of the active saccharide-utilizing community (3.2.4.2.1). Hence, the assay was optimized for the simultaneous detection of both taxa. The assays that either targeted the family *Planctomycetaceae* or the families *Micrococcaceae/Cellulomonadaceae* showed specificities of 100% (Table 26). Another combined assay was developed for targeting *Kineosporiaceae*, since a discrimination of the family *Nocardioideaceae* (also labeled in stable isotope probing experiments) was not possible. This assay had a specificity of almost 80% (Table 26) and amplified some non-target sequences related to the phyla *Acidobacteria*, *Cyanobacteria*, and *Firmicutes*. The assay for quantification of saccharolytic *Clostridiaceae* (Cluster I *Clostridiaceae*) yielded almost 85% of target sequences (Table 26) and only 3 of 19 clone insert sequences were related to *Pseudomonodaceae* (*Gammaproteobacteria*), a family that was detected in 16S

rRNA cDNA gene libraries of 'heavy' fraction but not identified as labeled taxon (3.2.4.2.2). Cloning of qPCR products amplified by primers specific for cellulolytic *Clostridiaceae* (Cluster III *Clostridiaceae*) revealed that none of the analysed sequences was related to sequences of this taxon (Table 26). In contrast to pure target DNA and non-target DNA for which the assay was specifically working, it was not possible to amplify any target sequence in environmental samples. Assays designed for quantification of the new family-level taxa 'Cellu1-3', 'Sphingo1-4' and 'Deha1' yielded 96%, 61%, and 80% (Table 26) specificity, respectively. Non-target sequences that were amplified by these assays were related to various organisms in the phyla *Cyanobacteria*, *Deltaproteobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Nitrospirae*.

Although published protocols were used for the quantification of total *Bacteria* and *Archaea*, the assays were checked for their specificity. The *Bacteria*-targeting primers were highly specific and did not yield any non-target taxon (i.e., sequences were not non-bacterial) (Table 26). In contrast, approximately 44% of the sequences that were amplified with the *Archaea*-targeting primers were non-archaeal (i.e., sequences were bacterial).

Table 26. Specificity of qPCR assays.

		Target sequences [%] ^a	Non-target sequences [%] ^a	Affiliation of non-targets (phylum, number of sequences)
Family-level assays				
1	<i>Micrococcaceae</i> & <i>Cellulomonadaceae</i>	100.0 (19)	0.0 (0)	---
2	<i>Kineosporiaceae</i> & <i>Nocardioideaceae</i>	78.9 (15)	21.1 (4)	<i>Acidobacteria</i> , 1 <i>Cyanobacteria</i> , 2 <i>Firmicutes</i> , 1
3	Cluster I <i>Clostridiaceae</i>	84.2 (16)	15.8 (3)	<i>Gamma</i> proteobacteria, 3
4	Cluster III <i>Clostridiaceae</i>	0.0 (0)	100.0 (20)	<i>Acidobacteria</i> , 6 <i>Actinobacteria</i> , 1 <i>Alphaproteobacteria</i> , 6 <i>Deltaproteobacteria</i> , 5 <i>Firmicutes</i> , 2
5	<i>Planctomycetaceae</i>	100.0 (20)	0.0 (0)	---
6	'Cellu1-3' ^b (<i>Bacteroidetes</i>)	95.5 (21)	4.5 (1)	<i>Cyanobacteria</i> , 1
7	'Sphingo1-4' ^b (<i>Bacteroidetes</i>)	61.1 (11)	38.9 (7)	<i>Deltaproteobacteria</i> , 3 <i>Gemmatimonadetes</i> , 4
8	'Deha1' ^b (<i>Chloroflexi</i>)	80.0 (16)	20.0 (4)	<i>Nitrospirae</i> , 2 <i>Firmicutes</i> , 2
Domain-level assays				
9	<i>Bacteria</i>	100.0 (20)	0.0 (0)	---
10	<i>Archaea</i>	55.6 (10)	44.4 (8)	<i>Actinobacteria</i> , 1 <i>Alphaproteobacteria</i> , 1 <i>Bacteroidetes</i> , 1 <i>Betaproteobacteria</i> , 2 <i>Firmicutes</i> , 1 <i>Planctomycetes</i> , 2

^a Parenthetical values are the number of sequences.

^b Name and affiliation as defined elsewhere (3.2.4.2.1).

3.5. Effect of Pesticides on the Degradation of Cellulose and Cellobiose

The pesticides Bentazon, Chlorothalonil, MCPA, Metalaxyl, and Nonylphenol were pre-screened for their potential to impact on the aerobic and anaerobic degradation of cellobiose (Figure 24).

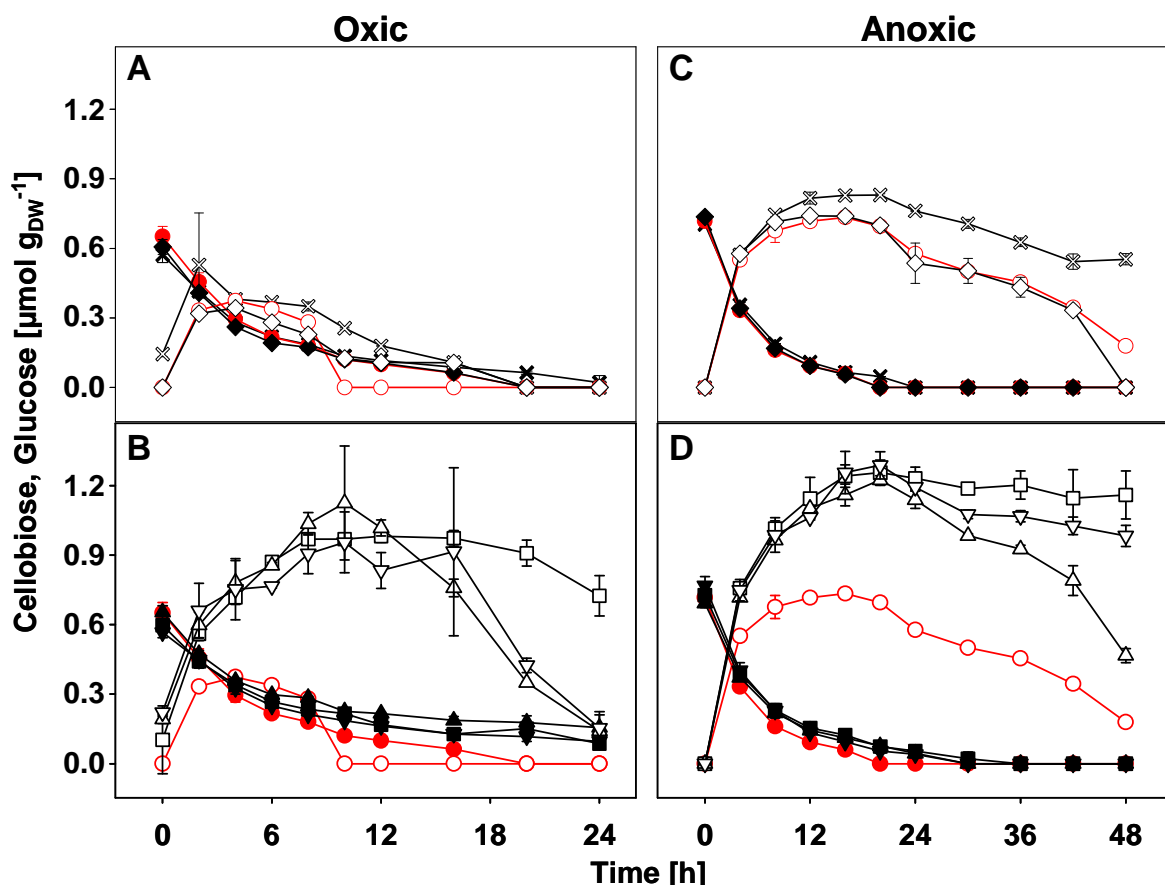


Figure 24. Degradation of supplemental cellobiose in soil treatments in the presence of pesticides. (A), (C): Pesticide without apparent effect (Chlorothalonil, Metalaxyl). (B), (D): Pesticide with apparent effect (Bentazon, MCPA, Nonylphenol). Symbols: ■ Bentazon-supplemented treatments; × Chlorothalonil-supplemented microcosm; ▼ MCPA-supplemented treatments; ◆ Metalaxyl-supplemented treatments; ▲ Nonylphenol-supplemented microcosms; ● Control treatments without pesticides (red). Closed symbols, cellobiose. Open symbols, glucose. Error bars indicate standard deviations (n =3).

Cellobiose was hydrolysed without apparent delay in oxic and anoxic treatments, resulting in an accumulation of glucose. Parallel to the hydrolysis of cellobiose that lasted 20 hours in pesticide-free control treatments, released glucose was consumed. Concentrations of glucose decreased in pesticide-free controls within 10 hours (oxic) and 48 hours (anoxic) to values under or near the detection limit ($\sim 0.5 \mu\text{mol g}_{\text{soil DW}}^{-1}$). Chlorothalonil and Metalaxyl did not apparently affect the aerobic and anaerobic degradation of cellobiose and the subsequent consumption of

glucose (Figure 24A and C). In contrast, the presence of Bentazon, MCPA, and Nonylphenol resulted in a slower hydrolysis of cellobiose and a strong inhibition of the subsequent consumption of glucose (Figure 24B and D). Complete cellobiose hydrolysis was delayed for 4 hours compared to pesticide-free controls. Furthermore, the concentration of free glucose doubled in Bentazon-, MCPA-, and Nonylphenol-supplemented treatments under oxic and anoxic conditions resulting in remaining glucose at the end of incubation (Figure 24B and D).

Based on these results, Bentazon, MCPA, and Nonylphenol were chosen as model pesticides to analyse the impact on the degradation of cellulose and cellobiose under oxic and anoxic conditions (Figure 25 and Figure 26; Table 27; Table A2, 2.3.3.3; 2.3.3.2). Different concentrations were applied to simulate *in situ* relevant ('low', 3.5.1) and elevated ('high', 3.5.2) concentrations of pesticides. Low amounts of pesticides were only used in soil slurries supplemented with cellobiose.

3.5.1. *In Situ*-relevant ('Low') Concentrations of Pesticides

Addition of cellobiose stimulated production of carbon dioxide by approximately 60% and small amounts of molecular hydrogen accumulated under anoxic conditions (Figure 25). Methane and ferrous iron were not detected in any of the treatments. pH was stable at 6.0 (data not shown). Supplemental cellobiose was hydrolysed without apparent delay in both oxic and anoxic treatments, leading to a transient accumulation of glucose (Figure 25). Both cellobiose and glucose were totally consumed within 10 hours under oxic conditions, and within 42 hours under anoxic conditions in control treatments lacking pesticides (Figure 25). Carbon recovery was 47% and 55% in oxic and anoxic control treatments, respectively. No apparent effect on the hydrolysis of cellobiose and the production of carbon dioxide was evident under 'low' concentration of pesticides (Figure 25; Table 27; Table A2), whereas a slight inhibition of the consumption of glucose occurred. Glucose was not totally consumed after 10 hours in oxic treatments supplemented with Bentazon or Nonylphenol (Figure 25) what refers to an inhibition of 12% and 25% by Bentazon and Nonylphenol, respectively (Table 27). Addition of pesticides to anoxic treatments affected consumption of glucose more pronounced than addition of pesticides to oxic treatments. Likewise, the total consumption of glucose in anoxic treatments was slower in the presence of pesticides than in pesticide-free control treatments what refers to an inhibition of 24 – 37% (Table 27). Production of molecular hydrogen under anoxic conditions was less in the presence of MCPA and negligible in the presence of Bentazon or Nonylphenol at 42 hours (Table A2).

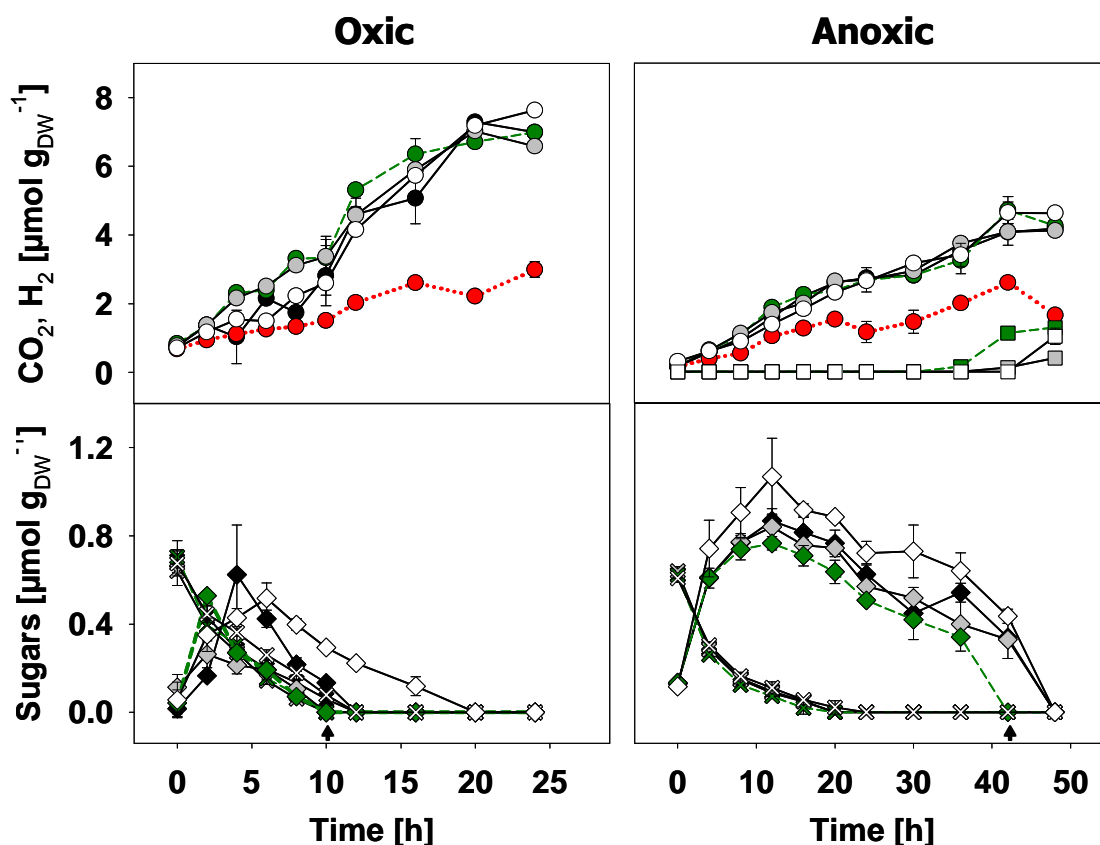


Figure 25. Degradation of supplemental cellobiose in treatments under 'low' concentration of pesticides. Pesticide concentrations: Bentazon: $0.1 \mu\text{mol g}_{\text{soil DW}}^{-1}$; Nonylphenol: $0.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$; MCPA, $0.01 \mu\text{mol g}_{\text{soil DW}}^{-1}$. Values are the means of duplicates. Error bars indicate standard deviations. Symbols: treatments supplemented with Bentazon (black), with Nonylphenol (white), with MCPA (grey), without pesticide supplementation (green, dashed line), or without substrate and addition of pesticides (red, dotted line). ● carbon dioxide; ■ molecular hydrogen; ✕ cellobiose; ◆ glucose. Arrows, time point for calculations of inhibitory effect of supplemented pesticides as presented in Table 27 and Table A2.

3.5.2. Elevated ('High') Concentrations of Pesticides

High concentrations of pesticides simulated the accumulation of pesticides that can occur in soil by heterogenous distribution (Marsh et al. 1978), especially in anoxic zones where pesticide degradation is slower compared to oxic zones. In treatments with higher concentration of pesticides cellobiose was not completely hydrolysed after 10 hours in oxic treatments (Figure 26), i.e., a reduction of the consumption of cellobiose of 38%, 38%, and 34% in the presence of Bentazon, MCPA, and Nonylphenol, respectively, was evident (Table 27). Likewise, the consumption of cellobiose-derived glucose and the production of carbon dioxide were lower in oxic treatments with high concentration of pesticides compared to treatments with low concentration of pesticides and controls lacking pesticides (Table 27). Between 0.3 ± 0.0 and $0.6 \pm 0.0 \mu\text{mol glucose g}_{\text{soil DW}}^{-1}$ were consumed after 10 days in oxic pesticide-supplemented treatments whereas the total amount of $1.4 \pm 0.1 \mu\text{mol glucose g}_{\text{soil DW}}^{-1}$ was consumed in the same period in

unsupplemented controls (Table A3). Production of carbon dioxide at high concentration of pesticides was almost as low as the soil indigenous production in substrate-free control treatments. In the presence of Bentazon and MCPA (Figure 26, Table A3) a reduction of 68% and 56% was measured under oxic conditions (Table 27). Nonylphenol showed only a slight effect on the production of carbon dioxide under oxic conditions (Table 27). Supplemental cellobiose hydrolysis was not affected in anoxic treatments supplemented with high concentration of pesticides (Figure 26), but a reduced consumption of glucose was observed in the presence of Bentazon and MCPA (Figure 26). At high concentration of pesticides, consumption of glucose was delayed for more than 12 hours, resulting in the detection of residual glucose at the end of incubation (Figure 26). The amount of utilized glucose was $0.2 \pm 0.1 \mu\text{mol g}_{\text{soil DW}}^{-1}$, and $0.3 \pm 0.0 \mu\text{mol g}_{\text{soil DW}}^{-1}$ in the Bentazon and MCPA-supplemented treatments, respectively (Table A3), what refers to a reduction of the consumption of glucose by more than 70% under high concentration of pesticides (Table 27). Interestingly, the initial anaerobic consumption of glucose was also slower under high Nonylphenol concentrations, but accelerated during incubation. This resulted in similar amounts of glucose consumed in both Nonylphenol-supplemented and unsupplemented control treatments (Figure 26, Table A3). Production of carbon dioxide at high concentration of pesticides was reduced by about 51 – 65% by MCPA and Bentazon, respectively (Table 27). No inhibition of the production of carbon dioxide or molecular hydrogen by Nonylphenol was obvious under anoxic conditions in which production of both gaseous compounds was stimulated, and concentrations exceeded control treatments at 42 hours (Figure 26). The anaerobic production of molecular hydrogen was negligible in the presence of Bentazon and MCPA (Table A3). Furthermore, the lack of fermentation products in these short incubations (Figure 24 – Figure 26; 3.5) demonstrates that strict anaerobes or facultative aerobes need time to form detectable amounts of molecular hydrogen, fatty acids, or alcohols after the depletion of O_2 . At high concentration of pesticides the pH immediately decreased to 5.4 ± 0.6 after the addition of pesticides, but stabilized at these value over the incubation period (data not shown).

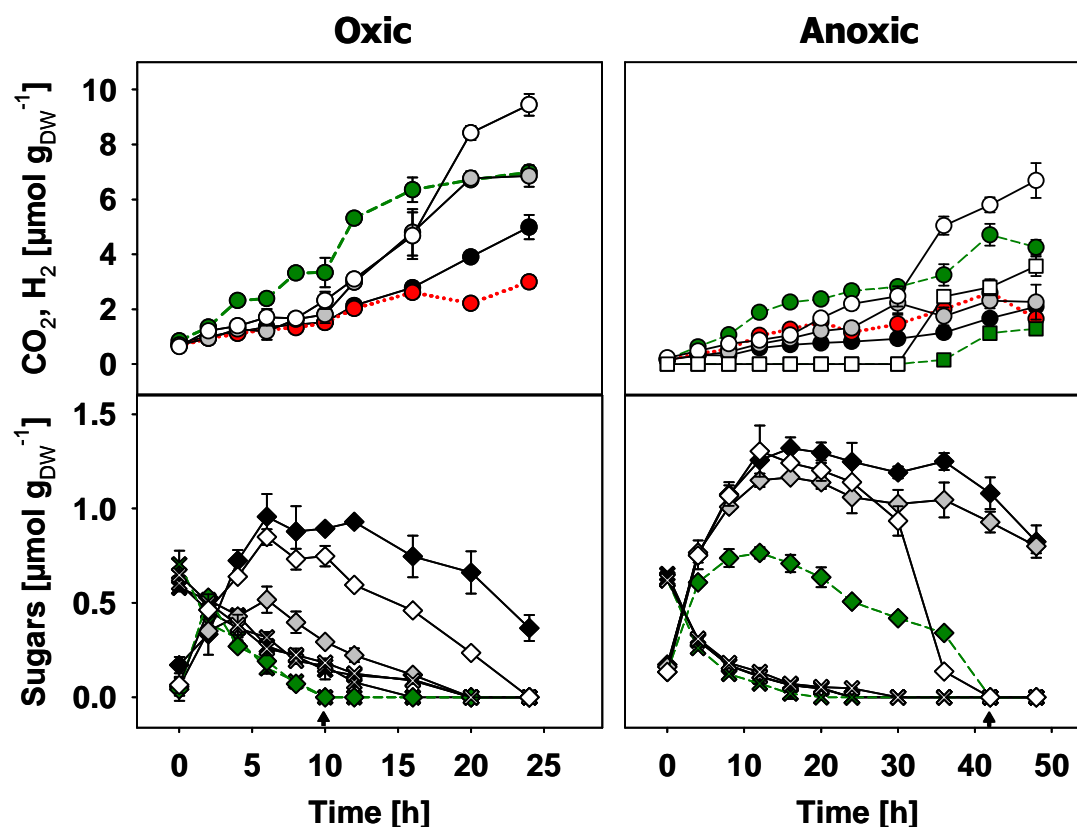


Figure 26. Degradation of supplemental cellobiose in treatments under 'high' concentration of pesticides. Pesticide concentration: Bentazon: $8.5 \mu\text{mol g}_{\text{soil DW}}^{-1}$; MCPA, $2.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$; Nonylphenol: $3.5 \mu\text{mol g}_{\text{soil DW}}^{-1}$. Values are the means of duplicates. Error bars indicate standard deviations. Symbols: treatments supplemented with Bentazon (black), with Nonylphenol (white), with MCPA (grey), without pesticide supplementation (green, dashed line), or without substrate and addition of pesticides (red, dotted line). ● carbon dioxide; ■ molecular hydrogen; ✕ cellobiose; ◆ glucose. Arrows, time point for calculations of inhibitory effect of supplemented pesticides as presented in Table 27 and Table A3.

The impact of pesticides on the degradation cellulose was investigated by insertion of cellulose paper sheets into sieved fresh soil and destructive sampling, i.e., removing of one replicate from the analysis, every 7 days. Approximately 50% of the supplemented cellulose was degraded during the 70-day incubation under both oxic and anoxic conditions in pesticide-free control treatments, i.e., about $12 \mu\text{mol cellulose g}_{\text{soil DW}}^{-1}$ was consumed (Figure 27, Table A3). Hydrolytic products of cellulose (cellobiose or glucose) were not detected in either oxic or anoxic treatments (detection limits approximated $0.5 \mu\text{mol g}_{\text{soil DW}}^{-1}$). Organic products were not detected during the first four weeks, but small amounts of acetate, propionate, and butyrate accumulated in anoxic cellulose-supplemented treatments ($< 5 \mu\text{mol g}_{\text{soil DW}}^{-1}$) lacking pesticides, but not in oxic treatments. Production of carbon dioxide was stimulated up to 50% by the addition of cellulose under both oxic and anoxic conditions without pesticides (Figure 27, Table A3). Carbon recovery was 106% and 81% in oxic and anoxic control treatments, respectively. Addition of cellulose also stimulated ferric iron reduction under anoxic conditions. Ferrous iron reached concentrations of up to about $190 \mu\text{mol g}_{\text{soil DW}}^{-1}$. In treatments without substrate, ferrous iron was not

detected (Figure 27, Table A3). Molecular hydrogen and methane were not detected in any of the cellulose-supplemented treatments. pH was stable at 6.2 (data not shown).

Bentazon, MCPA, and Nonylphenol at $2.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$ impaired the degradation of cellulose under oxic conditions by 41%, 62%, and 47%, respectively. Bentazon and MCPA reduced production of carbon dioxide by 20 – 32% (Table 27). Nonylphenol had no apparent inhibitory effect (Figure 27). In contrast, Nonylphenol stimulated production of carbon dioxide under oxic conditions (Table A3) what can be explained by the degradation of this pesticide. After 28 weeks $2.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$ were totally consumed in oxic treatments making a second application of Nonylphenol to remaining replicates necessary (Figure 27). Concentrations of Bentazon and MCPA decreased slowly in oxic cellulose-supplemented treatments, resulting in about 50% residual pesticide at the end of incubation (Figure 27).

Pesticide-impaired degradation of supplemental cellulose was more evident under anoxic than under oxic conditions, i.e. less than $1 \mu\text{mol cellulose g}_{\text{soil DW}}^{-1}$ was consumed (Figure 27, Table A3). This corresponds to an inhibition of more than 90% by all pesticides (Table 27). Accumulation of organic products (i.e., acetate, propionate, and butyrate) did not exceed $1.5 \mu\text{mol g}_{\text{soil DW}}^{-1}$ (data not shown) and production of carbon dioxide was impaired by 85%, 85%, and 44% for Bentazon, MCPA, and Nonylphenol, respectively (Table 27). Reduction of ferric iron was also reduced, i.e., 60 – 96% less ferrous iron accumulated in the presence of pesticides (Table A3). Concentration of pesticides was stable in anoxic cellulose-supplemented treatments (Figure 27).

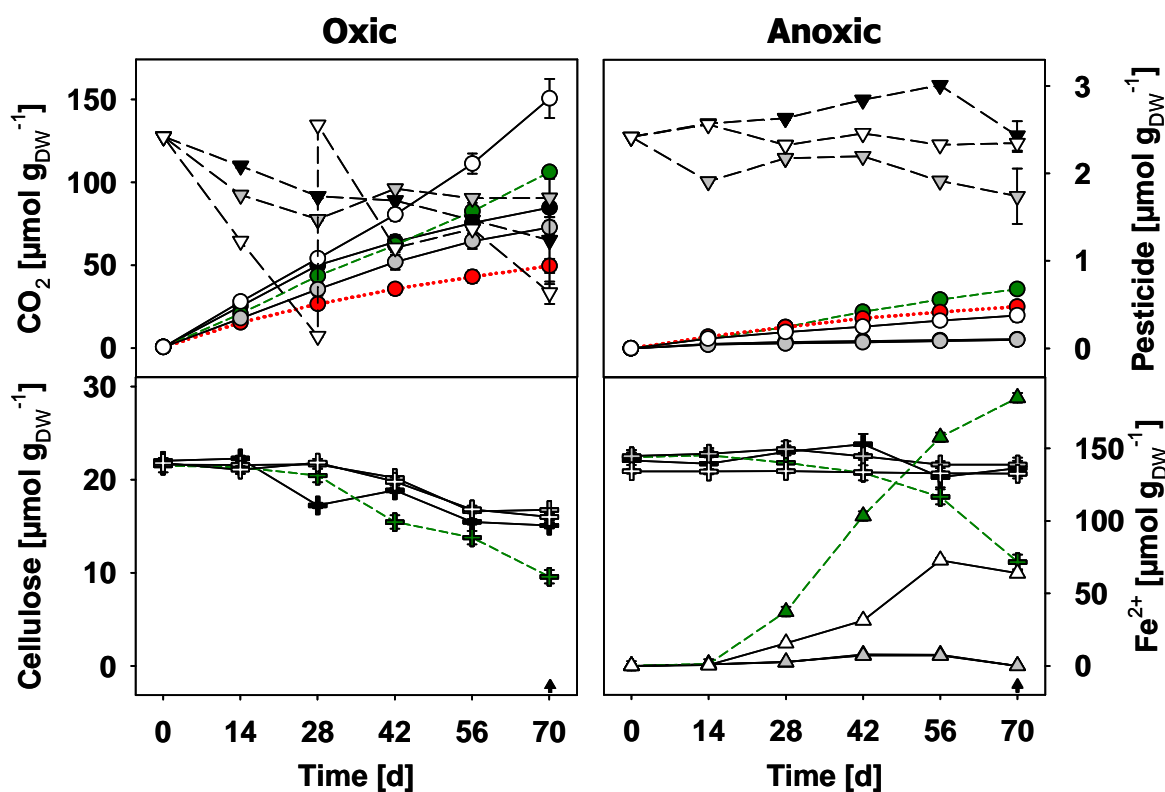


Figure 27. Degradation of supplemental cellulose in soil treatments in the presence of pesticides at $2.4 \mu\text{mol g}_{\text{soil DW}}^{-1}$. T_{end} values are the means of triplicates. Error bars indicate standard deviations. Symbols: treatments supplemented with Bentazon

(black), with MCPA (grey), with Nonylphenol (white), and without pesticide supplementation (green, dashed line), or without substrate and addition of pesticides (red, dotted line); ● carbon dioxide; ▼ pesticide (dashed line); + cellulose; ▲ ferrous iron. Arrows, time point for calculations of inhibitory effect of supplemented pesticides as presented in Table 27 and Table A3. Nonylphenol was applied twice (day 0, day 28) in oxic treatments.

Table 27. Inhibition of saccharide degradation, carbon dioxide and hydrogen emission, and production of ferrous iron in the presence of pesticides in soil treatments compared to control treatments.

	Inhibition [%]					
	Oxic			Anoxic		
	Bentazon	MCPA	Nonylphenol	Bentazon	MCPA	Nonylphenol
'Low' concentration of pesticides – Cellobiose-supplemented^a						
Cellobiose consumption ^d	11.1 ± 9.5	n.i.	15.4 ± 6.3	n.i.	n.i.	n.i.
Glucose consumption ^d	12.2 ± 6.3	n.i.	25.1 ± 8.4	26.6 ± 13.5	23.5 ± 2.5	37.3 ± 2.3
CO ₂ production ^d	n.i.	n.i.	n.i.	13.6 ± 11.3	13.4 ± 1.4	n.i.
H ₂ production ^d	-	-	-	100.0 ± 0.0	89.2 ± 1.5	100.0 ± 0.0
'High' concentration of pesticides – Cellobiose-supplemented^b						
Cellobiose consumption ^d	38.3 ± 7.0	37.8 ± 6.0	34.4 ± 0.3	n.i.	n.i.	n.i.
Glucose consumption ^d	81.0 ± 0.1	59.1 ± 4.3	61.1 ± 7.5	81.8 ± 6.0	73.4 ± 0.9	n.i.
CO ₂ production ^d	68.2 ± 3.3	55.6 ± 5.3	32.6 ± 2.4	64.7 ± 5.1	51.0 ± 5.9	n.i.
H ₂ production ^d	-	-	-	100.0 ± 0.0	100.0 ± 0.0	n.i.
'High' concentration of pesticides – Cellulose-supplemented^c						
Cellulose degradation ^e	40.8 ± 12.0	61.6 ± 2.6	47.4 ± 11.0	94.8 ± 5.0	93.8 ± 3.6	97.0 ± 3.4
CO ₂ production ^e	20.3 ± 6.5	31.5 ± 4.5	n.i.	84.6 ± 0.8	85.6 ± 2.1	44.2 ± 12.8
Fe ²⁺ production ^e	-	-	-	95.7 ± 0.5	95.4 ± 0.3	59.9 ± 1.0

^a 0.01 to 0.4 $\mu\text{mol pesticide g}_{\text{soil DW}}^{-1}$.

^b 2.4 to 8.5 $\mu\text{mol pesticide g}_{\text{soil DW}}^{-1}$.

^c 2.4 $\mu\text{mol pesticide g}_{\text{soil DW}}^{-1}$.

^d Inhibition of saccharide degradation, carbon dioxide and hydrogen emission was calculated based on the differences in the concentration of products at 10 hours (oxic) and 42 hours (anoxic) referred to the control treatment (Table A2).

^e Inhibition of cellulose degradation, carbon dioxide and hydrogen emission, and ferric iron reduction was calculated based on the differences in the concentration of products at 70 days of incubation referred to the control treatment (Table A3).

.i., No inhibition, i.e., amount of product was higher in pesticide-supplemented treatments than in control treatments or standard deviations where higher than the mean value.

-, Not determined.

3.5.3. 16S rRNA Content of Bacterial Taxa

16S rRNA transcript numbers of total soil *Bacteria* and five saccharide-utilizing family-level taxa (*Micrococcaceae/Cellulomonadaceae* [*Actinobacteria*], Cluster I *Clostridiaceae* [*Firmicutes*], *Planctomycetaceae* [*Planctomycetes*], and the new taxa 'Cellu1-3' and 'Sphingo1-4' [*Bacteroidetes*]), were determined in soil samples of cellulose-supplemented treatments using reverse transcriptase quantitative PCR (RTqPCR). Taxa were quantified in Bentazon-supplemented, MCPA-supplemented, and pesticide-free control treatments, but not in Nonylphenol-supplemented treatments.

RNA content of the total bacterial soil community decreased in all treatments from 6.4×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ to significantly lower values (Figure 28) independent of the presence or absence of pesticides. This consistent decrease might be explained by various factors, e.g., the death of non-saccharide-utilizing bacteria due to substrate limitation or grazing of *Bacteria* by eukaryotes feeding on bacteria (e.g., protozoa, ciliates). Bentazon and MCPA application lowered the number of transcript of total soil *Bacteria* in both oxic and anoxic treatments at the end of the experiment compared to pesticide-free treatments (Figure 28). RNA content decreased from 3.8×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ in pesticide-free controls to 1.5×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ in Bentazon-supplemented, and 1.2×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ in MCPA-supplemented treatments (Fig. 31) under oxic conditions. In anoxic treatments Bentazon and MCPA application lowered the number of 16S rRNA genes from 3.0×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ to 1.3×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$ and 0.6×10^{10} transcripts $\text{ng}_{\text{RNA}}^{-1}$, respectively (Figure 28).

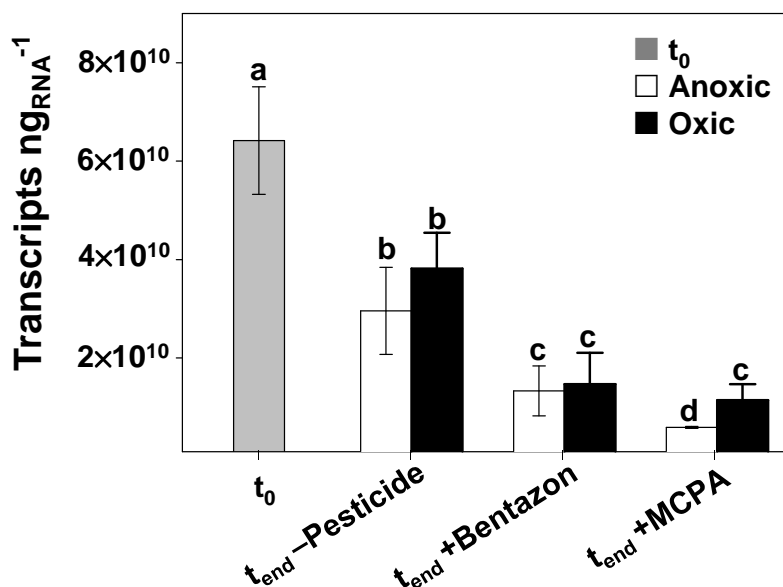


Figure 28. 16S rRNA gene transcript numbers of *Bacteria* in cellulose-supplemented treatments in the presence and absence of pesticides. Grey bar, value at the start of the experiment, i.e., the same value for the oxic and anoxic experiment. Black bars, oxic experiment. White bars, anoxic experiment. Error bars indicate standard deviations ($n = 3$). After was 70 days the experiment was terminated. Small letters indicates statistical differences of values ($p \leq 0.05$; t-test).

The taxa *Micrococcaceae/Cellulomonadaceae* were apparently not influenced by Bentazon or MCPA, i.e., transcript numbers did not change significantly from day 0 until the end of the experiment, neither under oxic nor under anoxic conditions (Table 28). Transcript numbers of *Planctomycetaceae* and uncultured 'Sphingo1-4' (phylum *Bacteroidetes*) were significantly lower after 70 days in oxic treatments with pesticide application compared to pesticide-free controls. Transcript numbers of *Planctomycetaceae* decreased in the presence of Bentazon and MCPA from 3.8×10^5 transcripts $\text{ng}_{\text{RNA}}^{-1}$ to 9.1×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$ and to 2.8×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$, respectively (Table 28). The new taxon 'Sphingo1-4' showed a decrease from 6.5×10^5 transcripts $\text{ng}_{\text{RNA}}^{-1}$ to 1.4×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$ in Bentazon- and to 2.0×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$ in MCPA-supplemented treatments (Table 28). Transcript numbers of both taxa were stable in pesticide-free controls (Table 28).

In contrast, transcript numbers of Cluster I *Clostridiaceae* and uncultured 'Cellu1-3' (phylum *Bacteroidetes*) were significantly lowered by pesticides under anoxic conditions (Table 28). RNA content of Cluster I *Clostridiaceae* decreased in the presence of Bentazon and MCPA from 1.3×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$ to 1.1×10^4 transcripts $\text{ng}_{\text{RNA}}^{-1}$ and to 7.0×10^3 transcripts $\text{ng}_{\text{RNA}}^{-1}$, respectively (Table 28). The new taxon 'Cellu1-3' showed a decrease from 5.0×10^2 transcripts $\text{ng}_{\text{RNA}}^{-1}$ to 3.0×10^1 transcripts $\text{ng}_{\text{RNA}}^{-1}$ in Bentazon-supplemented treatments. Interestingly, RNA content of 'Cellu1-3' increased in pesticide-free control and MCPA-supplemented treatments from day 0 to day 70, but the increase was significantly lower in MCPA-supplemented treatments compared to control treatments lacking pesticides (Table 28).

Table 28. 16S rRNA gene transcripts number of family-level taxa that are linked to cellulose degradation.

Taxon ^a	Transcript number [ng _{RNA} ⁻¹] ^b			
	0 days	- Pesticide	+Bentazon ^c	+MCPA ^c
		70 days		
Oxic				
<i>Micrococcaceae & Cellulomonadaceae</i>	$(4.6 \pm 1.5) \times 10^5$	$(3.4 \pm 0.8) \times 10^5$	$(1.7 \pm 0.7) \times 10^5$	$(1.9 \pm 0.9) \times 10^5$
Cluster I <i>Clostridiaceae</i>	$(1.3 \pm 0.2) \times 10^4$	$(8.3 \pm 1.8) \times 10^3$	$(6.5 \pm 3.9) \times 10^3$	$(2.0 \pm 1.4) \times 10^4$
<i>Planctomycetaceae</i>	$(3.8 \pm 0.3) \times 10^5$	$(2.3 \pm 0.6) \times 10^5$	$(9.1 \pm 2.6) \times 10^4$	$(2.8 \pm 0.9) \times 10^4$
'Cellu1-3'	$(5.0 \pm 0.7) \times 10^2$	$(4.3 \pm 1.3) \times 10^2$	$(4.3 \pm 1.2) \times 10^2$	$(5.2 \pm 3.2) \times 10^2$
'Sphingo1-4'	$(6.5 \pm 0.4) \times 10^5$	$(5.1 \pm 1.4) \times 10^5$	$(1.4 \pm 0.7) \times 10^4$	$(2.0 \pm 0.8) \times 10^4$
Anoxic				
<i>Micrococcaceae & Cellulomonadaceae</i>	$(4.6 \pm 1.5) \times 10^5$	$(2.5 \pm 0.9) \times 10^5$	$(2.7 \pm 1.3) \times 10^5$	$(1.4 \pm 0.6) \times 10^5$
Cluster I <i>Clostridiaceae</i>	$(1.3 \pm 0.2) \times 10^4$	$(4.3 \pm 1.2) \times 10^4$	$(1.1 \pm 0.4) \times 10^4$	$(7.0 \pm 2.0) \times 10^3$
<i>Planctomycetaceae</i>	$(3.8 \pm 0.3) \times 10^5$	$(7.8 \pm 5.9) \times 10^4$	$(9.8 \pm 3.9) \times 10^4$	$(4.2 \pm 0.1) \times 10^4$
'Cellu1-3'	$(5.0 \pm 0.7) \times 10^2$	$(9.7 \pm 5.3) \times 10^3$	$(3.0 \pm 0.6) \times 10^1$	$(6.5 \pm 1.6) \times 10^2$
'Sphingo1-4'	$(6.5 \pm 0.4) \times 10^5$	$(2.4 \pm 2.0) \times 10^4$	$(1.1 \pm 0.5) \times 10^5$	$(2.7 \pm 1.1) \times 10^4$

^a Name and affiliation of family-level taxa according to 2.5.14.1. A family was based on a minimal 16S rRNA gene similarity of 87% (Yarza et al. 2008).

^b Values are the mean of three replicates (with standard deviation). Bold numbers in pesticide-supplemented treatments are significantly different to pesticide-free controls (t-Test).

^c Concentration of pesticides was 2.4 $\mu\text{mol g}_{\text{soil DW}}^{-1}$.

3.6. Effect of Fluctuating Availabilities of O₂ on the Oxic and Anoxic Degradation of CMC and Cellobiose, and Evaluation of Metabolic Responses of Saccharide-utilizing Prokaryotes

Stable isotope probing experiments identified saccharide-utilizing bacteria that participated in the degradation of cellulose, cellobiose, and glucose under contrasting availabilities of O₂ (3.2.3.2). The response of major taxa to fluctuating availabilities of O₂ was determined in treatments supplemented with CMC or cellobiose (3.6.2). Furthermore, it was analysed how these taxa affected redox potentials during CMC and cellobiose degradation, and if *Archaea* were also an active constituent of the functionally redundant community.

3.6.1. Effect of the Degradation of CMC and Cellobiose on Redox Potentials, and the Formation of Degradation Products

Initial redox potential in oxic treatments, i.e., in CMC- or cellobiose-supplemented, and in unsupplemented redox controls, was 513 ± 5 mV (Figure 29 and Figure 30). Anoxic unsupplemented control treatments had an initial redox potential of 363 ± 3 mV that was relatively stable and decreased only slightly to 348 ± 12 mV (Figure 29). The redox potential of CMC-supplemented treatments was stable under oxic conditions, but decreased during the anoxic period to 324 ± 4 mV (Figure 30A). Redox potentials of cellobiose-supplemented treatments decreased rapidly within 10 hours after O₂ was exchanged by dinitrogen and remained constant until re-aeration (Figure 30B). Re-aeration with O₂ increased the redox potential rapidly within 2 – 4 hours to 600 ± 33 mV in CMC-supplemented treatments, to 411 ± 35 mV in cellobiose-supplemented treatments, and to 573 ± 25 mV in unsupplemented controls (Figure 31 and Figure 32).

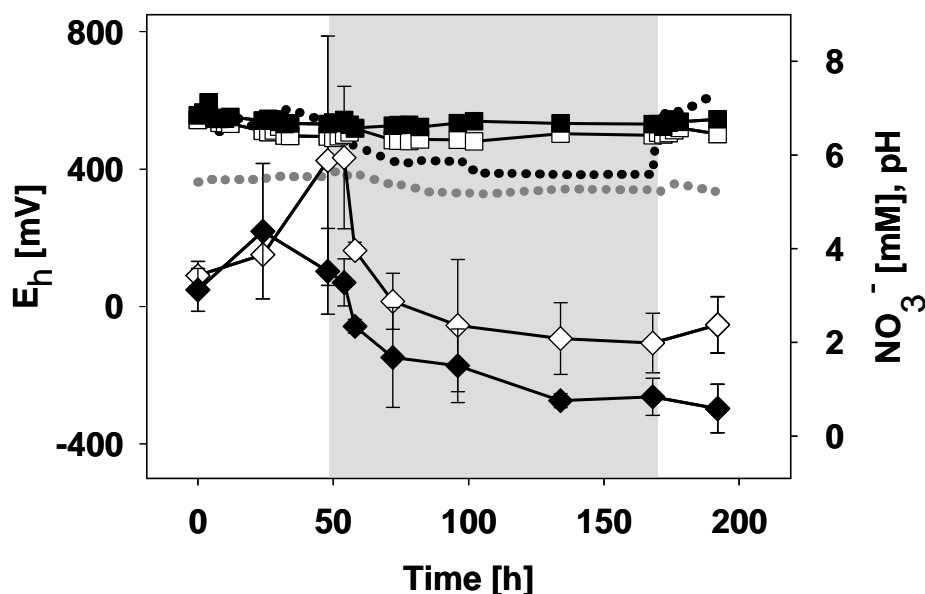


Figure 29. Redox potentials, concentration of nitrate, and pH of unsupplemented control treatments. The shaded area corresponds to the anoxic period. The empty symbols are values obtained from the unsupplemented treatments with changing O₂ availability, whereas the filled symbols are values obtained from the unsupplemented permanently anoxic treatments. Error bars indicate the standard deviation (n = 3). The black dotted line shows the redox potential of unsupplemented treatments with changing O₂ availability. The grey dotted line shows the redox potential of unsupplemented permanently anoxic treatments. Symbols: ◆, nitrate; ■, pH.

Concentrations of nitrate in CMC-supplemented treatments and unsupplemented redox controls increased during oxic phases, but slowly decreased during the anoxic phase (Figure 30A and Figure 29). This indicates that nitrate was formed by nitrification in the presence of O₂ and subsequently consumed anaerobically in the absence of O₂. In contrast, concentration of nitrate decreased linearly in anoxic controls, resulting in the detection of small amounts of nitrate (0.6 ± 0.5 mM) at the end of incubation (Figure 29). The majority of nitrate was consumed in cellobiose-supplemented treatments during the first oxic phase, suggesting that nitrate was reductively assimilated (Figure 30B).

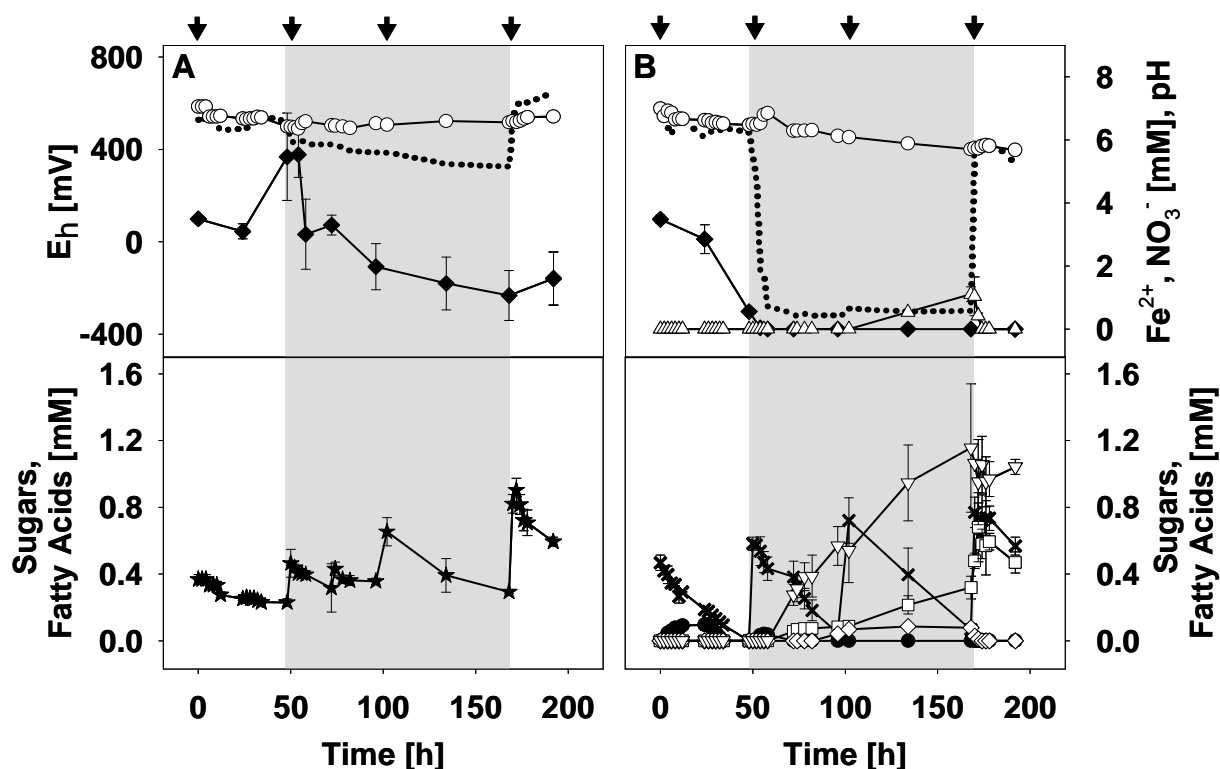


Figure 30. Concentration of substrate and products, redox potentials, and pH of (A) CMC-supplemented treatments or (B) cellobiose-supplemented treatments. The shaded areas correspond to the anoxic periods. The dotted line shows the redox potential. Symbols: ○, pH; ◆, nitrate; △, ferrous iron; ★, CMC; ×, cellobiose; ●, glucose; ▽, acetate; □, lactate; ◇, succinate. Error bars indicate their standard deviation ($n = 3$). The arrows indicate addition of substrates.

CMC and cellobiose were consumed without apparent delay at similar rates (CMC: $3 \pm 1 \mu\text{mol h}^{-1} \text{l}^{-1}$; cellobiose: $11 \pm 2 \mu\text{mol h}^{-1} \text{l}^{-1}$) subsequent to each pulse during both oxic and anoxic periods (Figure 30). Although concentration of CMC decreased, primary products of hydrolysis (e.g., cellobiose and glucose) were not detected, indicating that they were metabolized rapidly. This phenomenon was also observed in other experiments with cellobiose supplementation (3.1, 3.5). The formation of organic products and ferrous iron was also negligible during the anoxic period in CMC-supplemented treatments (Figure 30A). Cellobiose supplementation resulted in a transient accumulation of small amounts of glucose after the first and second pulse, whereas no glucose was detected at later time points (Figure 30B). The lack of glucose at later time points suggests that active microorganisms increased in cell numbers and/or consumed cellobiose more efficiently. Several fermentation products were formed after redox potentials decreased to values below -300 mV in cellobiose-supplemented treatments. Acetate, lactate, and succinate accumulated up to 1 mM during the anoxic phase (Figure 30B). Formate was produced in small amounts ($\sim 0.25 \text{ mM}$) during the first 48 hours of the anoxic phase, but was subsequently consumed during the latter part of the anoxic period (data not shown). Concentration of ferrous iron increased to 1 mM , suggesting that ferric iron was reduced (Figure 30B). Re-aeration resulted in the rapid decrease of ferrous iron and fatty acids (except lactate), indicating that these compounds were chemically

oxidized, dissimilated, or assimilated. The pH decreased from 6.6 ± 0.2 to 5.7 ± 0.0 in cellobiose- treatments, but was stable at approximately 6.6 in CMC and control treatments (Figure 30 and Figure 29).

3.6.2. 16S rRNA Content of Prokaryotic Taxa

The ribosomal content, i.e., transcript number, of the following microbial taxa was quantified with qPCR: *Archaea*_{Total}, *Bacteria*_{Total}, *Micrococcaceae/Cellulomonadaceae* (phylum *Actinobacteria*), *Kineosporiaceae/Nocardioideae* (phylum *Actinobacteria*), Cluster I *Clostridiaceae* (phylum *Firmicutes*), *Planctomycetaceae* (phylum *Planctomycetes*), the new taxa 'Cellu1-3' (phylum *Bacteroidetes*), 'Sphingo1-4' (phylum *Bacteroidetes*), and 'Deha1' (phylum *Chloroflexi*). QPCR assays (except *Archaea*_{Total} and *Bacteria*_{Total}) were designed in this study and evaluated for their specificity (3.4). 16S rRNA genes were quantified in DNA and cDNA, but family-level assays (Table 25) did not yield sufficient gene amplification in DNA samples (i.e., target sequences were below the detection limit of about 10 genes per reaction; $C_T > 30$). Therefore, results for all assays refer exclusively to transcript numbers quantified in cDNA samples.

CMC and cellobiose selectively stimulated the synthesis of bacterial and archaeal 16S rRNA in regard to the substrate, and the presence or absence of O_2 (Figure 31). CMC supplementation had no apparent effect on the synthesis of bacterial and archaeal 16S rRNA during the first oxic period, but stimulated production of rRNA of both domains in the second half of the anoxic incubation period (102 – 168 h) (Figure 31). Archaeal 16S rRNA increased significantly from 4.1×10^4 to 4.0×10^5 transcripts ng_{RNA}^{-1} , bacterial 16S rRNA increased from 7.2×10^7 to 4.0×10^8 transcripts ng_{RNA}^{-1} . Due to the low amount of target sequences amplified with the *Archaea*-specific primers (Table 26) it is likely that the observed increase of archaeal rRNA was caused by unspecific targeting of *Bacteria*. Re-aeration caused a slight decrease of bacterial and archaeal 16S rRNA gene transcripts in CMC-supplemented treatments to 2.1×10^8 and 2.3×10^5 transcripts ng_{RNA}^{-1} , a result linked to the potential death of strict anaerobes. Cellobiose supplementation resulted in the increase of bacterial and archaeal 16S rRNA from 0.6×10^9 to 5.0×10^9 transcripts ng_{RNA}^{-1} and 0.6×10^7 to 2.0×10^7 transcripts ng_{RNA}^{-1} , respectively, during the first oxic period (0 – 48 h; Figure 31). In contrast to CMC-supplemented treatments, the amount of bacterial and archaeal 16S rRNA transcripts was not altered by either the shift from oxic to anoxic conditions (48 – 168 h) or re-aeration (168 – 192 h) in cellobiose-supplemented treatments (Figure 31).

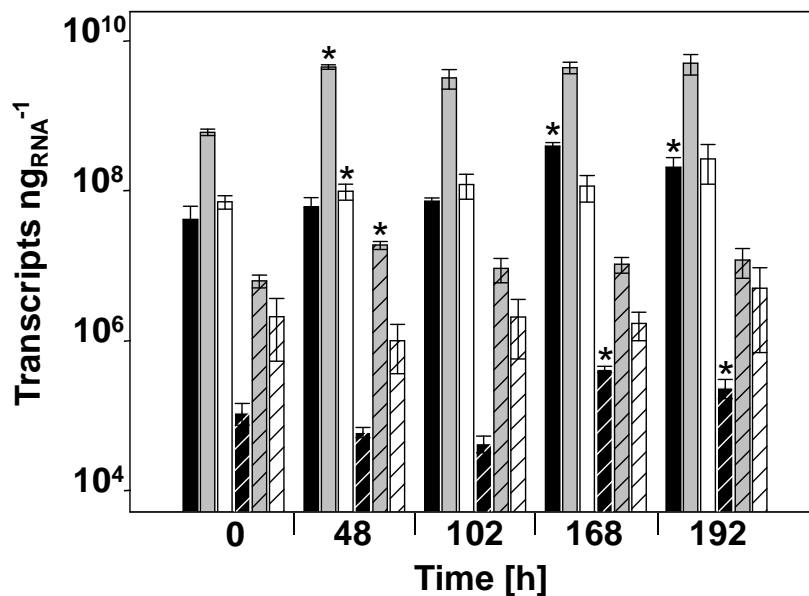


Figure 31. Numbers of 16S rRNA gene transcripts of *Bacteria* and *Archaea* in treatments in incubation chambers. The filled bars represent values obtained from CMC-supplemented treatments. The shaded bars represent values obtained from the cellobiose-supplemented treatments. The empty bars represent values obtained from unsupplemented control treatments. Error bars indicate standard deviations ($n = 3$). Bar code: non-hatched, *Bacteria*; hatched, *Archaea*. An asterisk indicates that a transcript number is statistically different from the transcript number of the previous time point (t-Test).

The synthesis of 16S rRNA of family-level taxa was differentially affected by addition of CMC and cellobiose (Figure 32 and Table A1). A selective activation and/or repression of functionally redundant taxa was observed (Figure 32). No family-level taxon was stimulated without addition of substrate, i.e., the amount of 16S rRNA genes did not change significantly in unsupplemented controls (Table A1). The relative amount of 16S rRNA of *Micrococcaceae/Cellulomonadaceae* increased significantly from 9.8×10^{-5} to 5.6×10^{-5} transcripts transcripts_{Bacteria}⁻¹ in the first oxic period of cellobiose-supplemented treatments, but not in CMC-supplemented treatments (Figure 32A, Table A1). Anoxic incubation (48 – 168 hours) resulted in a significant decrease of transcript numbers of *Micrococcaceae/Cellulomonadaceae* in cellobiose-supplemented treatments (Figure 32A). 16S rRNA gene transcript numbers of *Planctomycetaceae* behaved inversely (Figure 32D). Addition of cellobiose did apparently not affect RNA content of *Planctomycetaceae* (Figure 32D, Table 24), but addition of CMC resulted in a significant increase from 1.4×10^{-6} to 1.8×10^{-5} transcripts transcripts_{Bacteria}⁻¹ during re-aeration (Table A1). Members of *Kineosporiaceae/Nocardoidaceae* were not stimulated by either CMC or cellobiose (Figure 32B). In contrast, relative transcript numbers of this taxon decreased during the oxic and the first half of the anoxic period (0 – 168 hours) from 6.3×10^{-7} to 2.1×10^{-7} transcripts transcripts_{Bacteria}⁻¹ and from 9.3×10^{-7} to 1.3×10^{-7} transcripts transcripts_{Bacteria}⁻¹ in CMC- and cellobiose-supplemented treatments, respectively (Table A1). Similar results were obtained for the uncultured taxon ‘Deha1’ (phylum *Chloroflexi*), for which stimulation by addition of substrates was not apparent (Figure 32F, Table A1). Transcript numbers of Cluster I *Clostridiaceae* and the new family-level taxon ‘Cellu1-3’ (phylum *Bacteroidetes*) increased under anoxic conditions

(48 - 68 h) in cellobiose-supplemented treatments but not under oxic conditions (Figure 32C and E). Transcript numbers of Cluster I *Clostridiaceae* and 'Cellu1-3' increased to 1.9×10^{-7} and 1.0×10^{-7} transcripts transcripts_{Bacteria}⁻¹ after 168 hours of incubation in the presence of cellobiose, respectively (Table A1). CMC did not appear to stimulate these taxa, neither in the oxic period nor in the anoxic period (Figure 32C and E, Table A1). The uncultured taxon 'Sphingo1-4' (phylum *Bacteroidetes*) was the only taxon that was slightly stimulated by both substrates (Figure 32G, Table A1). In the second half of the anoxic period 16S rRNA genes increased from 8.4×10^{-9} to 2.2×10^{-8} transcripts transcripts_{Bacteria}⁻¹ and from 2.1×10^{-6} to 4.5×10^{-6} transcripts transcripts_{Bacteria}⁻¹ in CMC- and cellobiose-supplemented treatments, respectively (Table A1).

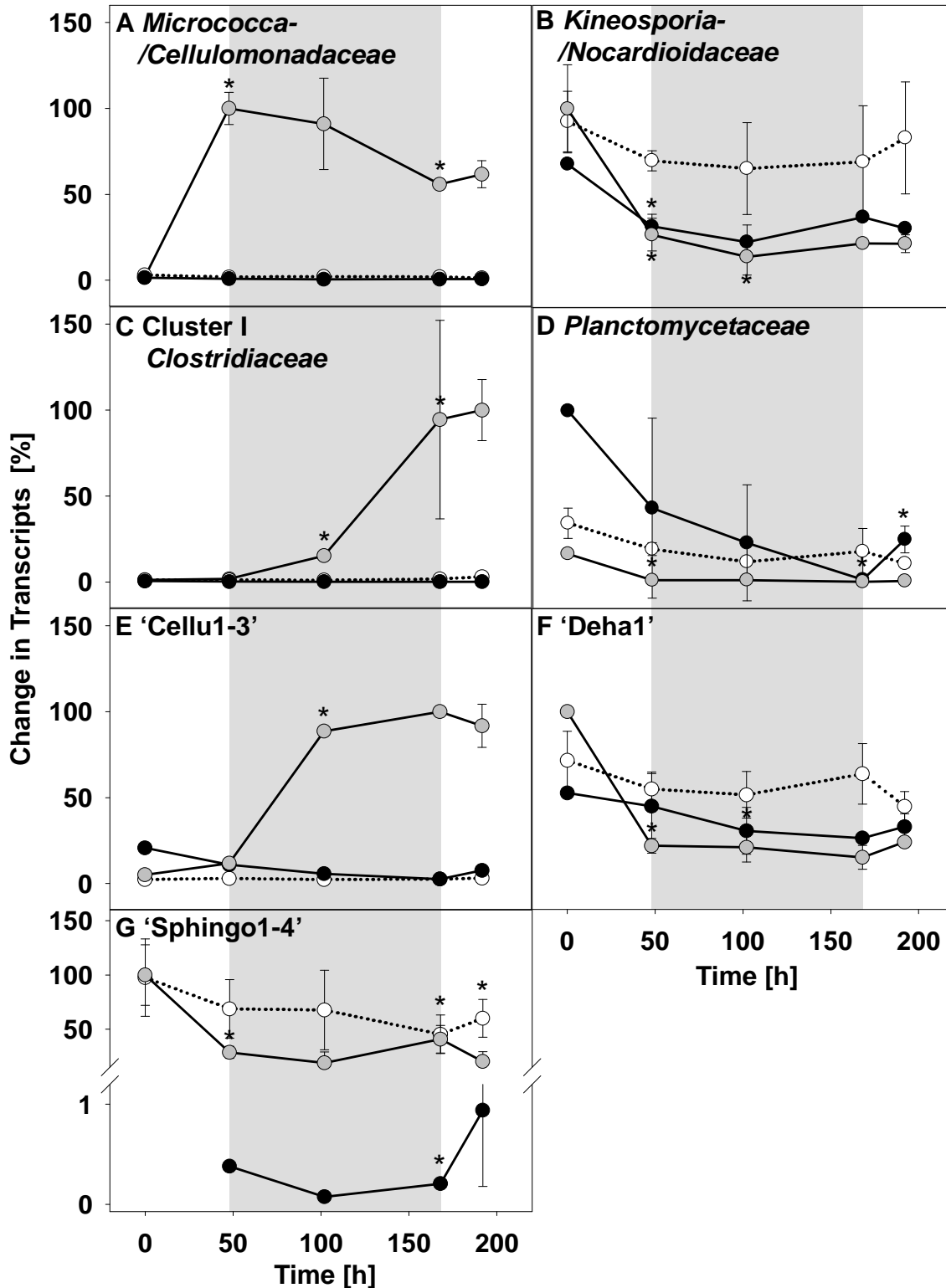


Figure 32. Effects of CMC and cellobiose on taxon-specific 16S rRNA gene transcripts. The shaded areas correspond to the anoxic periods. Symbols: filled, values obtained from CMC-supplemented treatments; grey, values obtained from cellobiose-supplemented treatments; empty, values obtained from unsupplemented control treatments. Values are the means of two replicates (without standard deviation) or three replicates (with standard deviation). The highest number of taxon-specific transcripts per assay (i.e., in CMC, cellobiose, and unsupplemented control treatments) was set at 100% (absolute and reference values are marked in Table A1). An asterisk indicates that a transcript number is statistically different from the transcript number of the previous time point (t-Test).

A shift in the ratio of family-level taxa was also observed. *Micrococcaceae/Cellulomonadaceae* became the most abundant of all detected groups in cellobiose-supplemented treatments after the initial oxic phase. Such an enrichment of rRNA of these *Actinobacteria* was not observed in CMC-supplemented treatments (Figure 33). In contrast, 16S rRNA cDNA genes of the new family-level taxon 'Deha1' (*Chloroflexi*) comprised a high proportion of the autochthonous soil microbial community (Figure 33), but neither this taxon nor other detected taxa were remarkably activated in CMC- or cellobiose-supplemented treatments (Figure 32).

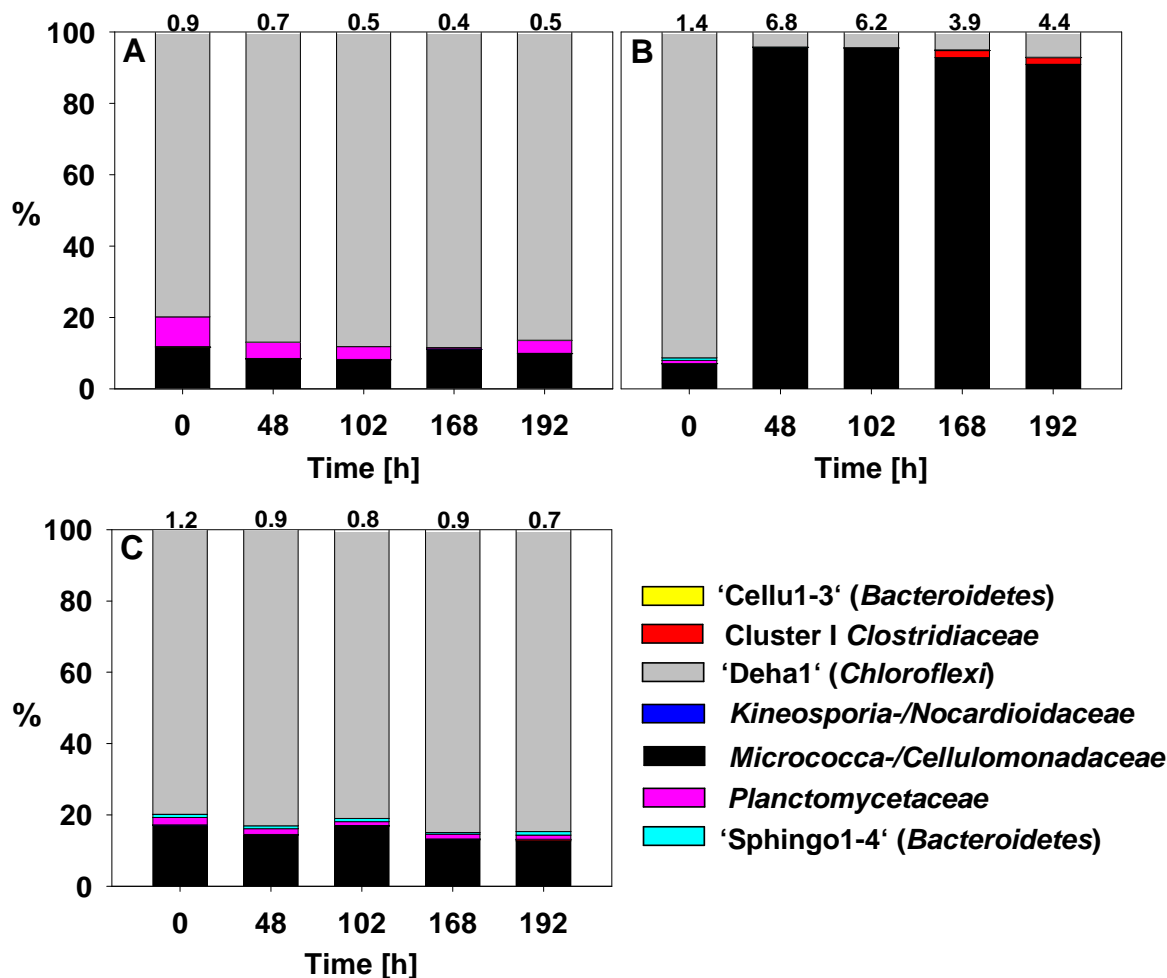


Figure 33. Relative abundances of family-level taxa in (A) CMC-supplemented treatments, (B) cellobiose-supplemented treatments, and (C) unsupplemented control treatments. 100% corresponds to the total number of transcripts of all taxa (numbers above bars in [$\times 10^{-3} \text{ transcripts ng}_{\text{RNA}}^{-1}$]).

4. DISCUSSION

Cellulose is the most abundant biopolymer on earth and its biological degradation in soil is a major process in the global carbon cycle (Bayer et al. 2006; Falkowski et al. 2000; Lal 2008; Lynd et al. 2002). Agricultural soils account for about 40% of the world's terrestrial surface area and are hot spots for the turnover of organic carbon (Körber et al. 2009). Agricultural soil is a highly structured environment in which oxic and anoxic microzones co-occur on a small scale (Figure 3), and in which the distribution and dimension of such zones can change rapidly (Dassonville et al. 2004; Or et al. 2007; Six et al. 2000; Totsche et al. 2010; Zausig et al. 1993). This compartmentalization facilitates the coexistence and simultaneous activity of aerobic and anaerobic soil microorganisms that catalyse the degradation of plant-derived cellulose under contrasting availabilities of O₂. However, less is known about the phylogenetic identities of cellulose-degrading microbial communities *in situ* and their metabolic response to rapid changes in O₂. Another crucial factor is the increasing usage of pesticides in agricultural ecosystems. Application of herbicides, fungicides, and insecticides has resulted in an accumulation of pesticide residues in terrestrial and aquatic habitats (Akerblom 2004; Hiller et al. 2008; Thorstensen et al. 2001). Pesticides can impact on soil communities (Cox et al. 1996; Johnsen et al. 2001; Katayama and Kuwatsuka 1991; Porter and Hayden 2002), and might alter the cellulose-dependent carbon flow and the activity of involved microbial taxa in agricultural ecosystems. Trophic interactions between cellulose-degrading microorganisms are complex and many uncultured species are likely important to the degradation of plant-derived carbon (Haichar et al. 2007; Li et al. 2009). To understand the process of cellulose degradation in such a dynamic environment, it is essential (i) to identify involved cellulose-degrading soil communities and (ii) to investigate the impact of O₂ and pesticides on active microbial taxa.

4.1. Aerobic and Anaerobic Degradation of Saccharides in An Aerated Agricultural Soil

A community function, e.g., the utilization of glucose or the turnover of nitrogen, may remain stable under changing O₂ states (Picek et al. 2000; Santruckova et al. 2004) although the composition of the microbial community shifts (Pett-Ridge and Firestone 2005; Pett-Ridge et al. 2006). In this regard, the capacity of the saccharide-degrading community to readily consume supplemental substrates was not affected by the availability of O₂ or changing redox potentials (Figure 11, Figure 27, Figure 30). Supplemented carboxymethyl-cellulose (CMC) was consumed without apparent delay in oxic and anoxic treatments (Figure 30A), indicating that the saccharide-degrading community of the soil was poised to both aerobically and anaerobically consume this substrate. In contrast to CMC-supplemented treatments, a short lag phase was observed in treatments that received cellulose in the insoluble form, i.e., cellulosic paper sheets (Figure 27). The lag phase before measurable degradation of cellulose paper suggests an initial colonization of the substrate by cellulolytic microorganisms prior to its biological degradation (Munier-Lamy and Borde 2000). A lower abundance of accessible regions for enzymatic hydrolysis in crystalline cellulose compared to soluble cellulose-derivates might also account for the slow degradation of cellulose paper. Endo- and exoglucanases excreted by cellulolytic microorganisms attack crystalline cellulose at internal amorphous regions, and increase the amounts of reducing and non-reducing chain ends (Bayer et al. 1998a; Bayer et al. 1998b; Bisaria and Ghose 1981; Lynd et al. 2002; Teeri 1997; Figure 2).

Thus, prolonged incubation promotes growth of cellulolytic microorganisms on cellulose sheets by an increase of substrate accessibility. The lack of detectable cellodextrins, cellobiose, and glucose during cellulose degradation (Figure 11A, Figure 27, Figure 30A) suggests a very efficient assimilation of released hydrolysis products by cellulolytic microorganisms and saccharolytic satellite organisms (Bayer et al. 1994; Bayer et al. 2004; Stanier 1942; Xie et al. 2007; Figure 4, 1.5), especially under oxic conditions. Under anoxic conditions, anaerobic cellulolytic microorganisms attack cellulosic substrates mainly by an extracellular enzyme complex (i.e., cellulosome; 1.2.1.2) that is covalently linked with the cell envelope and attaches tightly to the substrate by carbohydrate binding domains (Bayer et al. 1998b; Beguin and Aubert 1994; Desvaux 2005b; Lynd et al. 2002). Thus, the release of soluble hydrolysis products is minimal (Bayer et al. 1994; Beguin and Aubert 1994).

Cellobiose was hydrolyzed, resulting in a transient accumulation of glucose (Figure 11B, Figure 24 – Figure 26, Figure 30B). This phenomenon may have been caused by extracellular β -glucosidases that hydrolyzed cellobiose faster than glucose was consumed by microorganisms (Hong et al. 1981; Lynd et al. 2002). Cellobiose also accumulated in oxic stable isotope probing treatments with prolonged incubation (Figure 11B) what suggests a subsequent product-mediated inhibition of cellobiose hydrolysis/uptake by increasing concentrations of glucose (Corazza et al. 2005; Gong et al. 1977; Kajikawa and Masaki 1999). However, it remains unclear, why the hydrolysis of cellobiose was inhibited in oxic treatments of stable isotope probing experiment, but not in other experiments. Nevertheless, supplemented cellobiose and glucose were aerobically and anaerobically consumed. Carbon recovery was below 60% in oxic treatments supplemented with cellobiose or glucose (3.1, 3.5) which indicates that a substantial amount of carbon was assimilated during aerobic conversion. This trend is consistent with studies that assessed the incorporation of carbon from [^{13}C]- and [^{14}C]-labeled cellulose, cellobiose, or glucose into microbial biomass of forest, savannah, and loamy soil (DeForest et al. 2004; Fontaine et al. 2004; Schneckenberger et al. 2008). According to literature, carbon dioxide was the main end product of the degradation of supplemented saccharides under oxic conditions (Bayer et al. 2006; Beguin 1990, Beguin and Aubert 1994; Schmidt and Ruschmeyer 1958). These results demonstrate that processes that are linked to the aerobic degradation of saccharides are similar in various types of soil and might be catalyzed by microbial communities with similar ecosystem functions.

Carbon recovery was above 100% in anoxic treatments of stable isotope probing experiments (3.1) suggesting that supplemental substrates augmented the concomitant utilization of soil indigenous carbon and enhanced turnover of microbial biomass (Blagodatskaya and Kuzyakov 2008; de Nobili et al. 2001). This so-called 'Priming Effect' results in increased production of carbon dioxide due to stimulation of degradation of already available carbon compounds by soil microorganisms after addition of [^{13}C]-cellulose or other organic carbon compounds (Blagodatskaya et al. 2007; Blagodatskaya and Kuzyakov 2008; Fontaine et al. 2004; Zyakun and Dilly 2005). Products that are indicative of fermentative metabolisms accumulated under anoxic conditions (Figure 11, Figure 30), a result consistent with previous laboratory studies that have investigated the effects of O_2 limitation on aerated soils (Degelmann et al. 2009a; Küsel and Drake 1995). In flooded or wetland soils, cellulose, and its initial main breakdown products cellobiose and glucose are decomposed by anaerobes into fatty acids, alcohols, molecular hydrogen, and carbon dioxide, physiological events that can be coupled to acetogenesis and methanogenesis (Drake et al. 2009; Westermann 1996). However, hydrogenotrophic

acetogenesis (i.e., the hydrogen-dependent reduction of carbon dioxide to acetate by acetogens [Drake et al. 2006; Drake et al. 2008]) was likely of minor importance since the headspace was regularly flushed with dinitrogen (2.3.1). The production of acetate, butyrate, propionate, and molecular hydrogen (Figure 11, Figure 30, 3.1, 3.6.1) suggests that a combination of fermentation types was active under anoxic conditions, including mixed acid fermentation and *Clostridia*-typical propionate or butyrate fermentation (Buckel 2005; Gottschalk 1986; White 2007). These fermentative metabolisms are likely sources of molecular hydrogen (Buckel 2005; White 2007). In contrast to forest soils that can form huge amounts of succinate and ethanol via *Enterobacteriaceae*-facilitated mixed acid fermentation under anoxic conditions (Degelmann et al. 2009a), the agricultural soil investigated in the present study did not yield these products in significant amounts, suggesting that they were not main products of sugar fermentation under experimental conditions or subjected to consumption. This corresponds to the low relative abundance of active *Enterobacteriaceae* in anoxic treatments (Table 22). In contrast, addition of organic carbon to anoxic soil (i.e., rice field or fen soil) results in an immediate accumulation of molecular hydrogen, acetate, and propionate (Hamberger et al. 2008; Penning and Conrad 2007; Wüst et al. 2009). Interestingly, supplementation of cellobiose stimulated the production of lactate under anoxic conditions in treatments that were subjected to fluctuating availabilities of O₂ (2.3.2), and lactate was not rapidly consumed after re-aeration (Figure 30B). In contrast, lactate was not a detectable compound in stable isotope probing experiments (Figure 11; 3.1). Instead, propionate accumulated (Figure 11). Propionate is classically produced by strict anaerobic bacteria of the *Propionibacteriaceae* (*Actinobacteria*), *Veillonellaceae*, *Clostridiaceae*, and ‘*Streptopeptococcaeae*’ (*Firmicutes*; Graham et al. 2011). Propionate-producing bacteria can ferment glucose, alcohols, and some amino acids to propionate (Piveteau 1999), but their preferred substrate is lactate (Graham et al. 2011). Lactate can either be reduced via the acrylate pathway to acetate and propionate, or converted stepwise via the succinate-propionate pathway to succinate and propionate (Graham et al. 2011). Succinate can be an intermediate or an end product of *Propionibacteria*-related fermentation (Piveteau 1999). Neither succinate nor *Propionibacteria*-related sequences were detected in stable isotope probing experiments (Figure 11, Table 19, Table 20, Table 23) what suggests that the production of propionate was not catalyzed by *Propionibacteriaceae*. The lack of lactate and succinate during long anoxic incubation (up to 84 days in stable isotope probing experiments; 3.1) and their detection during shorter anoxic incubation (< 10 days; 3.6.1) indicates that these compounds are intermediates of the degradation of saccharides, are rapidly turned over, and likely function as precursors of propionate- and acetate-formation by the microbial soil community. Formate that was produced in traces during anoxic periods was also not a stable intermediate (data not shown). A similar route of carbon turnover is also supposed to be relevant in fen soil or in the gut of earthworms (Hamberger et al. 2008; Wüst et al. 2011).

Parallel to the degradation of supplemented saccharides nitrate was consumed in most oxic and anoxic incubations (Figure 29, Figure 30; 3.1, 3.6.1). This indicates that nitrate was subjected to assimilation and dissimilation. Aerobic and anaerobic assimilation of nitrate via nitrite (NO₂⁻) and ammonium (NH₄⁺) into biomass (Fuchs 2007; Rudolf and Kroneck 2005; Tiedje 1988), anaerobic dissimilation of nitrate to nitrite (DNR; Stolz and Basu 2002) or dinitrogen (denitrification; Payne 1981), and anaerobic dissimilation of nitrate to ammonium (DNRA; Fuchs 2007; Simon 2002) may account for the consumption of nitrate. The capability to assimilate nitrate is common for most bacteria, fungi, algae, and higher plants, and necessary to

incorporate nitrogen into cellular components (Rudolf and Kroneck 2005). In contrast, dissimilation of nitrate is catalyzed almost exclusively by *Prokaryotes* and is performed to gain energy under anoxic conditions (Tiedje 1988). Concentrations of nitrate increased in CMC-supplemented and unsupplemented treatments during the oxic period (3.6.1), and decreased under anoxic conditions (Figure 29, Figure 30). This suggests that the production of nitrate by nitrification exceeded the consumption of nitrate (Sprent et al. 1987; Yu et al. 2007), and that nitrate was anaerobically assimilated or utilized as electron sink by the soil community via dissimilatory pathways (Conrad 1996, Dassonville et al. 2004; Peters and Conrad 1996; Yu et al. 2007). Available nitrate might also account for the apparent lack of ferrous iron and fermentation product formation (Dassonville et al. 2004; Picek et al. 2000). Thermodynamically high redox potentials do not favor most fermentations and the reduction of nitrate or ferric iron (Bohn 1971). The reduction of nitrate and ferric iron in water-unsaturated bulk soil rather occurs at 230 mV and 150 mV, respectively (Mansfeldt 2004). Values of 300 mV (nitrate reduction) and 50 mV (ferric iron reduction) are measured in water-saturated rice field soil, respectively (Yu et al. 2007). Most fermentations (except lactate fermentation by aerotolerant lactic acid bacteria; Brioukhanov and Netrusov 2007; Chen et al. 2005; Matthies et al. 2004) occur optimally at redox potentials below -100 mV (Dassonville et al. 2004; Picek et al. 2000). Hence, the presence of nitrate in CMC-supplemented and unsupplemented treatments might have been responsible for the stabilization of redox potentials above 320 mV (Figure 29, Figure 30A).

In contrast to CMC- and unsupplemented treatments, a rapid decrease of the redox potential in cellobiose-supplemented treatments occurred when O₂ was removed. The redox potential dropped more than 700 mV within 10 h (Figure 30B). It is likely that this change in redox potential was due to the metabolic activity of saccharide-utilizing microbes that coupled the oxidation of saccharides to the reduction of high redox potential electron acceptors such as oxygen, nitrate, and ferric iron (Figure 11, Figure 30B; Dassonville et al. 2004; Küsel et al. 2002; Lovley et al. 2004; Peters and Conrad 1996). This conclusion is consistent with the labeling of strict anaerobic and facultative aerobic taxa capable of the reduction of nitrate or ferric iron. For example, species of *Cellulomonadaceae*, *Rhodocyclaceae*, *Clostridiaceae*, *Geobacteraceae*, *Pelobacteraceae* and *Aeromonadaceae* perform denitrification, reduce nitrate to nitrite or ammonium, or dissimilate ferric iron under anoxic conditions (Colwell et al. 1986; Kang et al. 2007; Lee 2006a; Lovley et al. 2004; Shida et al. 1997; Tiedje 1988; van Keulen et al. 2007; Wiegel et al. 2006). It is generally supposed that microorganisms that reduce ferric iron do not directly utilize complex organic matter (Lovley 2006; Lovley et al. 2004), but preferentially utilize fermentation products, such as fatty acids and alcohols, as electron donors (Lovley et al. 1993; Lovley et al. 1995). However, some iron reducers can also utilize glucose (e.g., *Aeromonadaceae*, *Clostridiaceae*; Dobbin et al. 1999; Pham et al. 2003). Hence, it can be assumed that species of *Aeromonadaceae* and *Clostridiaceae* catalyzed the saccharolytic reduction of ferric iron in the experiments.

Concomitant to the reduction of alternative electron acceptors with positive redox potentials and the high amounts of readily accessible organic carbon, the decreasing redox potential favored fermentation processes in cellobiose-supplemented treatments during the anoxic period (Figure 30B). Re-aeration of cellobiose-supplemented treatments resulted in a strong increase of the redox potential and fast consumption of fatty acids and ferric iron (Figure 30), the latter processes likely being mediated by heterotrophic aerobes.

Methane was not produced in any experiment under anoxic conditions. This result is in contrast to wetland soils that yield methane from saccharides via 'intermediary ecosystem metabolism' (i.e., by the trophically linked processes that precede methanogenesis [Drake et al. 2009]; 1.2.2). The production of methane in acidic fen soil can be stimulated by methanogenic precursors like formate or carbon dioxide, and non-methanogenic precursors like xylose or glucose (Hamberger et al. 2008; Hunger et al. 2011). This is coincident with the occurrence of active methanogens and typical fermenting species in fen soil, suggesting that fermentation processes and methanogenesis are trophically linked (Hamberger et al. 2008; Hunger et al. 2011). Furthermore, methanogens can be cultivated from aerated soils (Peters and Conrad 1995), and some species tolerate oxygen (Krätzer et al. 2011; Liu et al. 2008; Tholen et al. 2007). Methane production can occur in aerated soil samples if they are incubated for long periods (> 100 days) under anoxic conditions (e.g., Küsel and Drake 1996; Sexstone and Mains 1990; Vor et al. 2003). However, methanogens cannot utilize alternative electron acceptors. Thus, the primarily oxic nature of the investigated soil, the short incubation time, and the presence of alternative electron acceptors do not favor methanogenesis (Küsel and Drake 1994; Küsel et al. 1999).

Taking all this findings into account, it can be suggested that (i) the degradation of cellulose and cellulose-derived carbon in agricultural soil is independent of the availability of O₂ and (ii) that the activity of saccharide-utilizing soil microorganisms alters the soil redox potential.

4.2. Active Saccharide-Degrading Prokaryotic Communities

Different aerobic and anaerobic bacterial communities assimilated [¹³C]-cellulose and [¹³C]-cellulose-derived saccharides which resulted in the detection of 48 labeled family-level taxa (Table 22). The majority of these families had low relative abundances (< 3%) and were likely of minor importance for the degradation of cellulose (4.2.3). In contrast, some family-level taxa comprised higher relative abundances (> 3%) what suggests that these groups are cellulolytic and saccharolytic key taxa in the cellulose-linked carbon flow (4.2.1, 4.2.2).

Archaea were not labeled in the current study indicating that the experimental conditions did not favour the activity of archaeal community members, and that supplemented [¹³C]-saccharides were not assimilated by the archaeal soil community. This is in contrast to studies on fen soil and anoxic municipal waste landfill cover soil (Hamberger et al. 2008; Li et al. 2009; Wüst et al. 2009). *Crenarchaeota* are common in the environment and can dominate the ammonium-oxidizing community in agricultural soil (Aravalli et al. 1998; Gubry-Rangin et al. 2010; Leininger et al. 2006). Most abundant *Archaea* in aerated soils are non-methanogenic mesophiles of *Thaumarchaea* (Pester et al. 2011). These mesophilic organisms are generally assumed to be nitrifiers. Nonetheless, the largest proportion of the diversity of soil *Archaea* is physiologically uncharacterized (Bates et al. 2011; Pester et al. 2011). In some archaeal genomes cellulase genes were detected (Ando et al. 2002; Bauer et al. 1999), but known cellulolytic *Archaea* are extreme thermophiles (Graham et al. 2011; Perevalova et al. 2005; Robb et al. 2011). Thus, it is unlikely that cellulolytic *Archaea* play an important role in cellulose degradation in the investigated temperate soil community.

4.2.1. Key Taxa that Degraded Saccharides under Oxic Conditions

Although fungal growth on cellulose sheets was observed (data not shown) bacteria contributed to the degradation of cellulose sheets under oxic conditions (3.2.3.2.1). Cellulolytic and saccharolytic aerobic taxa detected in this study have been previously identified in and isolated from soil by the use of crystalline cellulose (Haichar et al. 2007; Lo et al. 2009; Patel and Vaughn 1973; Ulrich et al. 2008). In contrast, prokaryotic communities that degrade plant organic matter and leaves are dissimilar to active taxa of the current study (Bernard et al. 2007; Lee et al. 2011; Tanahasi et al. 2005). These differences might be based on the composition of plant litter. Plant cell walls consist of a lignocellulose complex, in which cellulose is embedded in lignin, hemicellulose and other polymers such as starch and proteins (Chang 2007; Kumar et al. 2008a; Malherbe and Cloete 2002). These polymers as well as other carbonaceous plant-derivates (e.g., photosynthetic products) are subjected to microbial mineralization (Boddy et al. 2007; Nultsch 2000; Six et al. 2004) and yield a broader diversity of soil organisms than pure cellulose. Bacterial communities responsible for the degradation of [¹³C]-labeled rice callus are dominated by saccharolytic members of *Gammaproteobacteria*, *Flavobacteria*, *Sphingobacteria* or *Acidobacteria* (Lee et al. 2011). Due to the low cellulose content of rice callus, active bacteria incorporate [¹³C]-carbon derived from water soluble organic compounds, but less from cellulose (Lee et al. 2011). Likewise, rice straw is mainly degraded by *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria*, *Bacteroidetes*, *Flavobacteria*, *Spirochaeta*, and *Verrucomicrobia* (Bastian et al. 2009; Bernard et al. 2007; Tanahasi et al. 2005).

Actinobacteria were the most labeled *Bacteria* in the stable isotope probing experiment and had highest abundances compared to other quantified saccharide-degrading taxa under oxic conditions. *Actinobacteria* comprise the majority of known aerobic cellulolytic isolates (de Boer et al. 2005; Lynd et al. 2002) and are one of the best studied phyla in the domain *Bacteria* (Stackebrandt and Schuhmann 2006). *Actinobacteria* include strict and facultative aerobic species that often build mycelium-like structures, i.e., hyphae (Eriskon 1949; Goodfellow and Williams 1983; Stackebrandt and Schuhmann 2006; Ventura et al. 2007). Hyphae-forming cellulolytic members may have an ecological advantage. By penetrating cellulosic material they achieve a better access to attackable regions and bring their cellulases in closer proximity to the substrate (Abdulla and El-Shatoury 2007; Chater et al. 2010; Eriksson et al. 1990; Lynd et al. 2002). Cellulolytic and saccharolytic *Actinobacteria* are often identified as parts of the bacterial community that is involved in the aerobic degradation of plant-derived carbon (e.g., Dumova and Kruglov 2009; Hofsten et al. 1971; Lee et al. 2011; Lo et al. 2009; Patel and Vaughn 1973; Tanahasi et al. 2005; Ulrich et al. 2008). Interestingly, cellulolytic *Actinobacteria* were not frequently labeled in oxic cellulose-supplemented treatments (Table 22, Figure A1). Only some sequences affiliated with *Cellulomonas composti*, a facultative cellulolytic bacterium of the family *Cellulomonadaceae* (Kang et al. 2007). This family consists of primarily aerobic species that are well known for their ability to degrade crystalline cellulose (An et al. 2005; Bagnara et al. 1985; Kang et al. 2007; Lo et al. 2009; Patel and Vaughn 1973; Ulrich et al. 2008). They are numerically important in soil and involved in the degradation of rice straw or the degradation of other plant organic matter (Lo et al. 2009; Stackebrandt and Keddie 1986; Stackebrandt et al. 2006; Tanahasi et al. 2005). The low coverage of the corresponding gene library (Table 21; 64%) might account for the low relative abundance of cellulolytic *Actinobacteria* that degraded crystalline cellulose under oxic conditions. With the

exception of *Cellulomonadaceae*, all detected actinobacterial families are incapable of growth on cellulose, and grow best aerobically by utilization of cellobiose and/or glucose (Goodfellow and Maldonado 2006; Hartmans et al. 2006; Jones and Collins 1986; Kudo et al. 1998; Lee 2006a; Lee 2006b; Padmanabhan et al. 2003; Pagani and Parenti 1978; Stackebrandt and Schuhmann 2006; Stackebrandt et al. 2006; Tao et al. 2004; Yoon et al. 2004; Yoshimi et al. 1996). Nevertheless, the appearance of non-cellulolytic *Actinobacteria* in cellulose-supplemented treatments suggests that this phylum harbors so far unknown cellulolytic members that belong to known or new families that may contribute to degradation of cellulose in aerated soil. Despite the low abundance of cellulolytic *Actinobacteria*, saccharolytic members were frequently detected. *Micrococcaceae* was the most frequently labeled family in cellobiose- and glucose-supplemented treatments, followed by *Intrasporangiaceae*, *Kineosporiaceae*, and *Nocardioideae* (Table 22). These taxa might have cellulolytic potentials and some are supposed to act as saccharolytic satellite microorganisms in a cellulose-utilizing sub set of the soil community (Hartmans et al. 2006; Patel and Vaughn 1973; Ulrich et al. 2008). A higher proportion of *Intrasporangiaceae* was detected in glucose than in cellobiose treatments (Table 22) which fits well to knowledge of substrate preference of pure cultures of *Intrasporangiaceae* (Lee 2006b). Enrichment from soil inocula with cellulose and cellobiose as growth substrates often yield *Actinobacteria* (Lo et al. 2009; Patel and Vaughn 1973; Ulrich et al. 2008). The high number of labeled saccharolytic *Actinobacteria* (Table 19) and their stimulation by cellobiose (Figure 32A) further suggest that members of this phylum, especially *Micrococcaceae*-, *Cellulomonadaceae*- and *Intrasporangiaceae*-related species, are important potential degraders of plant-derived saccharides in the investigated soil.

Bacteroidetes-related taxa, i.e., 'Sphingo1-4', were also frequently [¹³C]-labeled in oxic cellulose-supplemented treatments (Table 22, Figure A3). The family-level groups 'Sphingo1-4' were stimulated by both cellobiose and CMC, even under anoxic conditions (Figure 32G; 4.4). However, the 'Sphingo1-4'-targeting qPCR assay detected additionally two non-target families, i.e., *Gemmatimonadaceae* (*Gemmatimonadetes*) and *Polyangiaceae* (*Deltaproteobacteria*) (Table 26). These families include some species that are capable of aerobic growth on cellulose (Lampky 1971; Zhang et al. 2003). Nevertheless, the strictly aerobic phenotype of the non-target taxa suggests that the significant increase in 'Sphingo1-4'-affiliated rRNA in the anoxic period was caused by active anaerobic species of *Sphingobacteriales*. The capacity to utilize cellulose and cellulose-derived saccharides is common to various members of *Bacteroidetes* (Bernardet et al. 1996; Nakagawa and Yamasato 1996; Reichenbach 2006; Robert et al. 2007), and cellulolytic enrichment cultures can be dominated by cellulose-degrading *Bacteroidetes* (Hofsten et al. 1971). The next related cultivated organism of 'Sphingo1-4' (86% 16S rRNA gene similarity) was the cellulolytic species *Cytophaga hutchinsonii* (Louime et al. 2006). It is likely that 'Sphingo1-4' represent new cellulolytic taxa that are in part capable of being active under both oxic and anoxic conditions.

Planctomycetaceae might also be an important aerobic saccharide-degrading family in aerated soils. *Planctomyces* are ubiquitous in many environments and can comprise 4 – 12% of bacterial rRNA in agricultural soils (Buckley and Schmidt 2003; Ward et al. 2006). *Planctomyces* are often isolated from aquatic carbon-rich habitats such as acidic bogs or sewage treatment plants (Ward et al. 1995). Described *Planctomycetaceae* do not utilize cellulose, but some species may be involved in the turnover of particulate organic matter (e.g., *Sphagnum* moss material; Kulichevskaya et al. 2007; Tadonleke 2007). *Planctomycetaceae* incorporated [^{13}C] from cellulose, but not from cellobiose or glucose (Table 19, Table 22), and showed a retraded but clear metabolic response to CMC supplementation (Figure 32D, 4.4). All 16S rRNA gene sequences that were amplified with the *Planctomycetaceae*-specific qPCR assay belonged to the genus *Gemmata* (Franzmann and Skerman 1984). *Gemmata*-related species were isolated from leakage water of a composts heap and soil (Wang et al. 2002; Ward et al. 1995), but their contribution to the cycling of carbon in soil has not been resolved. Hence, it remains unclear if *Planctomycetaceae* inhabit so far unknown cellulolytic species or if they act as satellite organisms during the degradation of cellulose, but obviously they are involved in carbon flux from plant organic matter.

'Deha1' (*Chloroflexi*) might also represent a new cellulolytic group due to its labeling in cellulose-supplemented treatments (Table 19, Table 22). Although *Dehalococcoidetes* have not been found to be cellulolytic, cellulolytic *Chloroflexi* have been isolated from compost, rotting wood, or sewage (Garrity and Holt 2001; Lee and Reichenbach 2006; Yabe et al. 2010). Members of the *Chloroflexi* can also be numerically important in cellulose-fed biofilm communities (Ishii et al. 2008), excrete glycosidic exoenzymes (Kragelund et al. 2007), and contribute to the degradation of [^{13}C]-labeled rice straw and polysaccharides in activated sludge samples (Kragelund et al. 2007; Lee et al. 2011). Known pure cultures of this metabolically versatile phylogenetic group are filamentous, and comprise photo- and chemoheterotrophs, aerobes and anaerobes (Garrity and Holt 2001). *Chloroflexi* are typical constituents of bacterial communities in freshwater or marine ecosystems, but their role in soils is largely unresolved (Garrity and Holt 2001). The new family-level taxon 'Deha1' was not stimulated by either CMC or cellobiose (Figure 32B and F), although species of this new family might be capable of assimilating carbon derived from [^{13}C]-cellulose (Table 19, Table 22). Hence, the contribution of the family-level taxon 'Deha1' to the degradation of cellulose remains unclear.

4.2.2. Key Taxa that Degraded Saccharides under Anoxic Conditions

Cellulolytic and saccharolytic *Firmicutes* were mainly responsible for the degradation of cellulose and cellulose-derived carbon under anoxic conditions (Table 20, Figure A2; 3.2.4.2.1). *Clostridiaceae* was the most frequent taxon in anoxic treatments followed by members of *Actinobacteria* and new family-level taxa of *Bacteroidetes* (i.e., 'Cellu1-3'; Table 22). Some labeled taxa from cellulose-supplemented treatments are also identified by other studies as key groups for the degradation of cellulose in permanently anoxic environments (Table 29).

Table 29. Bacterial key organisms that are involved in the degradation of cellulose in permanently anoxic environments.

Anoxic environment	Key organisms [phylum: family] ^a	Reference
Rumen	<i>Acidobacteria: Fibrobacteraceae</i> <i>Bacteroidetes: Bacteroidaceae</i> <i>Firmicutes: Clostridiaceae, Lachnospiraceae, Ruminococcaceae</i>	Kamra 2005; Russell et al. 2009
Anoxic soil/sediment	<i>Firmicutes: Clostridiaceae, Ruminococcaceae</i>	Li et al. 2009; Uz and Ogram 2006; Uz et al. 2007
Cellulose-fed anaerobic enrichments	<i>Alphaproteobacteria:</i> <i>Hyphomicrobiaceae</i> , <i>Rhizobiaceae</i> <i>Bacteroidetes: Bacteroidaceae</i> <i>Chloroflexi: Anaerolinaceae</i> <i>Firmicutes: Clostridiaceae</i>	Ishii et al. 2008; Ozkan et al. 2001; O'Sullivan et al. 2005; O'Sullivan et al. 2007
Guts of humans/invertebrates (i.e., termites, earthworms, springtails, isopoda, and millipedes)	<i>Actinobacteria: Cellulomonadaceae, Corynebacteriaceae, Microbacteriaceae, Micrococcaceae, Micromonosporaceae, Streptomycetaceae</i> <i>Alphaproteobacteria: Bradyrhizobiaceae, Brucellaceae, Rhizobiaceae</i> <i>Bacteroidetes: Bacteroidaceae, Cytophagaceae</i> <i>Betaproteobacteria: Alcaligenaceae</i> <i>Firmicutes: Clostridiaceae, Bacillaceae, Paenibacillaceae</i> <i>Gammaproteobacteria:</i> <i>Enterobacteriaceae</i>	Chassard et al. 2010; König 2006

^a Taxa that are labeled in cellulose-supplemented treatments in the current study are printed in bold letters.

Cluster I and III *Clostridiaceae* are frequently isolated from composts and organic material-rich soil (Figure A2; Mallet et al. 2004; Ohmiya et al. 2005; Ozkan et al. 2001; Petitdemange et al. 1984). The production of several fermentation products (Figure 11) correlates well with their metabolic capabilities. Saccharolytic species yield large amounts of molecular hydrogen, carbon dioxide, acetate, and butyrate during the fermentation of saccharides (Mountfort et al. 1997; Rainey et al. 2009). Lactate and propionate can also result from fermentation activities of saccharolytic species (Rainey et al. 2009; Wiegel et al. 2006). Cellulolytic species do not produce

butyrate (Wiegel et al. 2006) what is in accordance with the lack of this fatty acid in cellulose-supplemented treatments (Figure 11A; Uz and Ogram 2006). The significance of *Clostridiaceae* is further reflected in their numerical importance in cellulose-fed digesters (Ishii et al. 2008; O'Sullivan et al. 2005; Ozkan et al. 2001) and anoxic sediments (Uz and Ogram 2006).

'Cellu1-3' were also major taxa labeled in cellulose-supplemented treatments (Table 20, Table 22). Cellulolytic species of *Bacteroidetes* are widely distributed in terrestrial habitats (Reichenbach 2006; Stanier 1947; Xie et al. 2007), and are important degraders of plant-derived polysaccharides in human and animal gut ecosystems, e.g., in ruminants (Dodd et al. 2011; Kamra 2005). Although sequences of 'Cellu1-3' did not cluster closely to cultivated species (< 87% 16S rRNA gene similarity), the next cultured organisms belong to *Cytophagaceae* (4.2), a family that has the capacity to utilize cellulose and cellulose-derived saccharides, and that is present in soils (Murray et al. 1984; Nakagawa and Yamasato 1996; Reichenbach 2006). The detection of 'Cellu1-3' in anoxic cellulose-supplemented treatments and the stimulation of this taxon by cellobiose in the anoxic period (Figure 32E) is further consistent with (i) the fermentative cellulolytic phenotype of the phylum which they were affiliated with (Robert et al. 2007), (ii) the capacity of cellulolytic *Bacteroidetes* of landfill soil to assimilate carbon from [¹³C]-cellulose (Li et al. 2009), and (iii) that *Bacteroidetes* are also numerically important in cellulose-fed digesters (Ishii et al. 2008; O'Sullivan et al. 2005; O'Sullivan et al. 2007). Thus, 'Cellu1-3' likely represent new phylogenetically deep-branching groups of cellulolytic anaerobes.

These collective findings demonstrate that different trophic guilds of *Firmicutes* (especially *Clostridiaceae*) and *Bacteroidetes* are not only key organisms for the degradation of cellulose and soluble sugars in permanently anoxic environments, but can persist also in well-aerated soils (e.g., by the formation of endospores; Park et al 2007) and might become activated if these soils are exposed to anoxic conditions.

Paradoxically, many sequences labeled in anoxic cellulose-supplemented treatments were affiliated to *Kineosporiaceae* (Table 22, Figure A1). To date cultivated species of *Kineosporiaceae* only grow aerobically and are incapable of hydrolysis of cellulose (Kudo et al. 1998; Pagani and Parenti 1978; Yoon et al. 2004). The high frequency of *Kineosporiaceae*-affiliated sequences in gene libraries from anoxic cellulose-supplemented treatments suggests that new species capable of anaerobic metabolism occur in this family. However, this taxon was not stimulated by either CMC or cellobiose (Figure 32B and F). Hence, it remains unclear if *Kineosporiaceae*-related species were directly involved in the degradation of cellulosic material and its hydrolysis products or if they use conversion products of these saccharides.

4.2.3. Labeled Taxa that are of Minor Importance for the Degradation of Saccharides

Further family-level taxa without cultured representatives were labeled in oxic and anoxic [¹³C]-supplemented treatments, but with minor abundance in gene libraries and TRFLP patterns (Table 22). The new taxa 'Micro3' and 'Micro2' might represent actinobacterial saccharolytic families as they were only detected in oxic glucose or cellobiose treatments. New family-level taxa of *Alphaproteobacteria*, i.e., 'Rhizo1' and 'Rhizo2', were labeled in oxic cellulose-supplemented treatments. The production of cellulases by species of *Rhizobium* is essential for the nodulation of plant roots (Robledo et al. 2008), and *Rhizobiales* are important saccharolytic

members in cellulose-fed biofilm communities and during the degradation of [^{13}C]-labeled cellulose (Dumova and Kruglov 2009; Haichar et al. 2007; Ishii et al. 2008). 'Myxo1', 'Desu1', 'Burk1', 'Gam1', 'Prot1', and 'Bac2' also lack cultured representatives. Higher-rank taxa related to these families include strict aerobic and anaerobic bacteria that are often associated with decaying wood and rotten organic material (Kersters et al. 2006; Kohno et al. 2002; Kuever et al. 2005).

Clostridial aerobiosis is unknown, but a new family-level taxon of *Clostridiales*, i.e., 'Clos3', was labeled in oxic cellulose treatments (Table 19, Table 22). The ability to degrade cellulose is characteristic for certain clostridial cluster (Collins et al. 1994; Uz et al. 2007; Weber et al. 2001) and some members can deal with microoxic conditions (Karnholz et al. 2002; Kawasaki et al. 2004; Kawasaki et al. 2005; Küsel et al. 2001). Hence, 'Clos3' might consist of new cellulolytic species that are less sensitive to O_2 . Vice versa, *Streptomycetaceae* were labeled in cellulose-supplemented treatments under anoxic conditions, although soil-borne cellulolytic species of *Streptomyces* are considered as strict aerobes (Kim et al. 2003; Lynd et al. 2002). *Streptomyces coelicolor* survives periods of anoxia (van Keulen et al., 2007) and expresses enzymes that are involved in the degradation of cellulose under these conditions (Langlois et al. 2003). Likely hitherto undiscovered cellulolytic and facultative aerobes exist in this family, and thus, *Streptomycetaceae* may be important for degradation of cellulose under both oxic and anoxic conditions.

Other less abundant taxa incorporated [^{13}C]-carbon from cellulose under anoxic conditions (Table 22). *Intrasporangiaceae*, *Hyphomicrobiaceae*, *Paenibacillaceae*, and *Rhodocyclaceae* are strict or facultative aerobes, but are not known to contain cellulolytic species (Garrity et al. 2005; Lee 2006b; Pason et al. 2006; Urakami et al. 1995). However, *Hyphomicrobiaceae* seem to be relevant for the degradation of organic material in cellulose-fed digesters (Ishii et al. 2008) and some species of *Paenibacillaceae* hydrolyse cellulose derivatives (Pason et al. 2006). 'Bac1' and 'Bac3' were distantly related to taxonomically classified organisms and may represent new cellulolytic *Bacteria*. *Aeromonadaceae*, *Enterobacteriaceae*, 'Gam1' and 'Clos4' assimilated [^{13}C]-carbon in anoxic cellobiose or glucose treatments. The corresponding phyla include facultative aerobes and strict anaerobic species that ferment saccharides (Fischer-Romero et al. 1996; White 2007). The capacity of *Aeromonadaceae*, and *Enterobacteriaceae* in fen and forest soils to assimilate carbon from supplemented glucose under anoxic conditions has also been documented (Degelmann et al. 2009a; Hamberger et al. 2008).

Interestingly, lactate occurred in cellobiose treatments, even under oxic conditions (Figure 30B). In contrast to clostridial lactate production, classical lactate fermentation can occur in the presence of O_2 and is catalyzed by aerotolerant anaerobes, i.e., lactic acid bacteria (Brioukhanov and Netrusov 2007; Chen et al. 2005; Matthies et al. 2004). Lactic acid bacteria are not capable of direct fermentation of cellulose, but some *Lactobacillus* species can use cellobiose as carbon source (Adsul et al. 2007; Carr et al. 2002; Okano et al. 2010). Lactic acid bacteria are essential for human health and play an important role in food industries (Carr et al. 2002; Okano et al. 2010). Although known soil-inhabiting lactic acid bacteria (e.g., *Lactococcus* and *Lactovum* [(Brioukhanov and Netrusov 2007; Matthies et al. 2004)]) were not detected with molecular methods in the current study, it is likely that lactic acid bacteria of the investigated soil microbial community also consumed cellulose-derived saccharides under hypoxic conditions that do not prefer other fermenters, such as the *Clostridiaceae*. In summary, these low-abundant families contributed to the aerobic and anaerobic degradation of cellulose-derived

hydrolysis products, but are likely no key groups for the cellulose-linked carbon sequestration in the investigated agricultural soil under laboratory conditions.

4.2.4. A Large Uncultured Sub-Community is Selectively Activated during the Aerobic and Anaerobic Degradation of Cellulose

Currently, 204 taxonomically valid bacterial families have been published (<http://www.bacterio.cict.fr/number.html#total>; last visit: 04.10.2011). The present study detected 26% of all validated bacterial families which corresponds to 52 described family-level taxa (Table 22, Table 23; 4.2, 4.2.2). Forty-eight bacterial families were labeled in [¹³C]-supplemented cellulose, -cellobiose and -glucose treatments (Table 22, 4.2) of which 28 did not closely affiliate with known species and represent new family-level taxa. Especially, the phyla *Bacteroidetes* and *Chloroflexi* were represented exclusively by taxa without cultured representatives, and together accounted for 7 new-family level taxa (Table 22). These findings illustrate that the hitherto unknown diversity of microorganisms that are associated with the decomposition of cellulosic biomass is large. Further research is necessary to resolve the role of minor abundant and new family-level taxa during the aerobic and anaerobic degradation of cellulose in soils.

In contrast to other studies, *Proteobacteria* were of minor importance for the degradation of [¹³C]-derived carbon in the current study (Table 22). *Proteobacteria*, especially *Alpha-* and *Gammaproteobacteria*, are widely distributed in almost every type of environment (Kerstens et al. 2006, Zhang and Xu 2008) and are frequently identified as part of the active cellulose-degrading community in insect guts (Adams et al. 2011; Morales-Jiminez et al. 2009) and soil (e.g., An et al. 2005; Bastian et al. 2009; Bernard et al. 2007; Haichar et al 2007; Lee et al 2011; Ulrich et al. 2008). Some *Proteobacteria* exhibit hydrolytic activity against soluble cellulose-derivates, e.g., CMC, but not on crystalline cellulose (Kerstens et al. 2006; Patel and Vaughn 1973; Ulrich et al. 2008). They further utilize a wide range of saccharides and thus, are supposed to act as saccharolytic satellite organisms during the degradation of cellulose and cellulosic substrates (Bernard et al. 2007; Dumova and Kruglov 2009; Ishii et al. 2008, Patel and Vaughn 1973; Tanahasi et al. 2005). However, only 2%, 7%, and 11% of labeled 16S rRNA sequences were related to *Proteobacteria* in [¹³C]-glucose, -cellobiose, and -cellulose treatments, respectively (data not shown). In contrast, *Actinobacteria* and *Firmicutes* dominated 16S rRNA gene libraries with relative abundances of 30 – 59% and 19 – 54%, respectively (data not shown). It is possible, that *Proteobacteria* were outcompeted by other taxa that are well adapted to utilize complex substrates such as cellulose (Bastian et al. 2009; Lynd et al. 2002). Hence, bacterial taxa other than *Proteobacteria* (i.e., *Actinobacteria* and *Firmicutes*, and as yet uncultivated taxa) are likely key groups of the cellulose-degrading community in the investigated agricultural soil.

Another interesting result of the current study was that the presence or absence of O₂ activated different bacterial communities (4.2.1, 4.2.2). Labeled taxa in glucose and cellobiose treatments were also dissimilar to those of the cellulose treatment, suggesting that experimental conditions selected for different community members. This finding correlates with previous studies. For example, differences in active species that belong to *Clostridia* have been observed between [¹³C]-cellulose- and [¹³C]-glucose-supplemented incubations with landfill soil (Li et al. 2009). Saccharolytic clostridial species of Cluster I, IV, and XIV comprise active parts of the soluble saccharide-degrading community in anoxic sediments whereas the

cellulolytic activity is linked to species of Cluster IV and XIV (Uz and Ogram 2006). Anaerobic cellulolytic *Clostridia* can be limited to a range of carbohydrates (Li et al. 2009; Lynd et al. 2002), growing best on cellulose and cellodextrins, but showing reduced growth on cellobiose and glucose (Ng and Zeikus 1982; Zhang and Lynd 2005). For example, *Clostridium thermocellum* has bioenergetic benefits by growth on cellulose-derived cellodextrins which is a result of the lower energy input when cellodextrins are taken up, and are then intracellularly cleaved as compared to up take of cellobiose and glucose (Zhang and Lynd 2005). Growth of the anaerobe *Bacteroides cellulosolvens* is only supported by cellulose and cellobiose, but not by glucose or other carbon sources (Murray et al. 1984). Aerobic cellulolytic species can also have different preferences towards cellulose and soluble sugars. Molar growth yields of *Actinotalea fermentans* (Yi et al. 2007) are higher on cellulose compared to glucose (Bagnara et al. 1987). Another point that might account for the observed differences is the tight attachment of the cellulosome of cellulolytic anaerobes to cellulose (Bayer et al. 1994a; Bayer et al. 2006). As a consequence, the release of hydrolysis products, e.g., cellobiose or glucose, is minimal (Bayer et al. 1994a; Bayer et al. 2006) what was presumably growth-limiting for saccharolytic satellite organisms in anoxic cellulose-supplemented treatments. Supplementation of soluble saccharides might have further favored saccharolytic organisms rather than cellulolytic ones. Saccharolytic bacteria are well adapted to rapidly utilize cellobiose or glucose (Lynd et al. 2002), but certain cellulolytic species, e.g., *Sporocytophaga myxococcoides*, need time for adaptation to soluble sugars (Sjipstein and Farhaeus 1949). Hence, an out-competition of cellulolytic taxa by saccharolytic taxa in cellobiose- and glucose-supplemented treatments is likely.

These conclusive results suggest that (i) a large uncultured diversity of soil *Bacteria* was involved in the degradation of cellulose and products of its hydrolysis, and (ii) the active saccharolytic community differed phylogenetically from the active cellulolytic community under and between oxic and anoxic conditions (Hypothesis 1, 1.7).

4.2.5. Degradation of Cellulose is a Stable Community Function in the Investigated Soil

The distribution of oxic and anoxic microzones in aerated soils is highly dynamic and may change rapidly (1.6.1). It is conceivable that both the degradation of cellulose and the active saccharide-degrading community is impacted by fluctuations of the availability of O₂, e.g., after precipitation events. However, there was no apparent delay in the consumption of supplemented saccharides during oxic and anoxic periods (Figure 30). Other functions of the soil microbial community are also not dependent on the availability of O₂ and the redox potential. For example, although glucose consumption rates in aerated soil can vary in response to O₂, the capacity of the microbial community to consume glucose is independent of redox potential (Picek et al. 2000; Santruckova et al. 2004). Also, the availability of O₂ does not appreciably affect nitrogen turnover rates in tropical forest soil, even though the presence or absence of O₂ engages different taxa of the microbial community that are involved in nitrogen turnover (Pett-Ridge and Firestone 2005; Pett-Ridge et al. 2006). Likewise, selective activation of aerobic and anaerobic saccharide-degraders under contrasting availabilities of O₂ allowed for a continuous degradation of cellulose in this study (Figure 32). *Micrococcaceae* and *Cellulomonadaceae* were the taxa that responded most pronounced to the supplementation of saccharides under oxic conditions (Figure 32A). A slight stimulation was also evident for *Planctomycetaceae* and the

new family 'Sphingo1-4' (*Bacteroidetes*) during re-aeration (Figure 32D and G). Cluster I *Clostridiaceae* and the new *Bacteroidetes*-related taxa 'Sphingo1-4' and 'Cellu1-3' responded positively under anoxic conditions (Figure 32C, E and G). Thus, it can be concluded that the rapid metabolic response of functionally redundant taxa, i.e., taxa that have the capacity to utilize the same substrate under contrasting environmental conditions (e.g., oxic and anoxic), contributes to the stability of a particular function of the microbial community despite differing availabilities of O₂ (Hypothesis 2, 1.7).

4.3. Diversity of Metabolic Active but not [¹³C]-Labeled Bacteria

Thirty-five of 83 detected family level-taxa were not labeled in stable isotope probing experiments (Table 23; 3.2.3.2). RNA stable isotope probing was applied, because it is more advantageous than DNA or PLFA (phospholipid fatty acid) stable isotope probing (Boschker et al. 1998; Manefield et al. 2002a; Radajewski et al. 2000). RNA is synthesised at higher rates than DNA, labeling of RNA occurs without cellular replication, and RNA provides sequence information that allows a higher phylogenetic resolution than PLFA analysis (Manefield et al. 2002a; Manefield et al. 2002b; Radajewski et al. 2003). Furthermore, DNA persists in the environment after cell death and cannot be easily used to define the metabolic state (i.e., active or inactive) of the organism it belongs to (Coolen and Overmann 1998). In contrast, RNA is labile and degrades rapidly after cellular inactivation or cell death (Kramer and Singleton 1993; Lee and Kemp 1994). Hence, the RNA-based detection of non-labeled families indicates that these taxa were metabolically active (Mengoni et al. 2005; Urich et al. 2008).

The majority of non-labeled taxa belonged to the phyla *Actinobacteria* (21.1%), followed by little abundant taxa of *Deltaproteobacteria* (6.0%), *Betaproteobacteria* (5.2%) and *Alphaproteobacteria* (3.9%), *Gammaproteobacteria* (2.0%), *Firmicutes* (1.9%), *Bacteroidetes* (1.4%), *Acidobacteria* (1.1%), *Chloroflexi* (0.5%), *Verrucomicrobia* (0.5%), and *Fusobacteria* (0.1%, Table 23). Detected non-labeled actinobacterial families can aerobically and anaerobically utilize a wide range of carbon compounds present in the organic fraction of soil (e.g., sugars, starch, pectin) or are able to grow autotrophically (Chen et al. 2004; Evtushenko and Takeuchi 2006; Jackson et al. 1995; Kim et al. 2007; Kim et al. 2008; Lynd et al. 2002; Lu et al. 2010; Stackebrandt and Schumann 2000; Tamura et al. 1999; Wade et al. 1999). Cellulolytic activity within non-labeled *Actinobacteria* is only reported for *Micromonosporaceae* (Gallagher et al. 1996; Lynd et al. 2002; Wilson 1992). Families of the phylum *Bacteroidetes* and of the phylum *Deltaproteobacteria* include members of the so-called 'gliding bacteria', i.e., non-flagellated bacteria that are highly motile due to surface-associated movement (Nett and König 2007). Some gliding bacteria are important degraders of cellulose in soil (Nett and König 2007; Reichenbach 2006) or have a predatory lifestyle (Kerstens et al. 2006). *Acidobacteria*, *Firmicutes*, and *Gammaproteobacteria* represent also phyla with cellulolytic members (Humphry et al. 2003; Kim 1995; Kumar et al. 2008a) that can be parts of the rumen ecosystem in which they primarily ferment amino and fatty acids (Cook et al. 1994; van Gylswyk 1995; Janssen and O'Farrell 1999). Cellulolytic *Acidobacteria* are slow-growing constituents of the plant-decomposing community in *Sphagnum* peat, but were likely out-competed by more efficient utilizers of plant-derived carbon, e.g., *Cytophagaceae* species (Eichhorst et al. 2011; Pankratov et al. 2008; Pankratov et al. 2011).

The remaining family-level taxa lack described cellulolytic species (Table 23), but some process cellulase genes (Reinhold-Hurek et al. 1993; Robledo et al. 2008), or are parts of the cellulose-degrading community in soil and cellulose-fed enrichments (e.g., *Anaerolineaceae*, *Alcaligenaceae*, and '*Xiphinematobacteriaceae*'; Dumova and Kruglov 2009; Haichar et al. 2007; Ishii et al. 2008; Patel and Vaughn 1973). It is supposed that most of these families act as satellite organisms and secondary utilizers of cellulose-derived degradation products (Dumova and Kruglov 2009; Hofsten et al. 1971; Ishii et al. 2008). Only 30% of the ribosomal RNA of an organism has to be labeled until it can be detected by RNA stable isotope probing (Jehmlich et al. 2008). The large number of non-labeled families might indicate that these *Bacteria* incorporated amounts of [¹³C]-carbon of less than 30% into their RNA, assimilated soil indigenous carbon, or fed on other bacteria. The latter two assumptions are supported by the enhanced turnover of soil indigenous carbon as reflected in imbalanced carbon recovers under anoxic conditions (4.1) and the physiological capabilities of these family-level taxa, which include predation and saccharide utilization.

4.4. Diversity of Active Eukaryotes

The capability to degrade cellulose is also a wide spread trait of microbial *Eukarya* (1.3.1) and different eukaryotic families were labeled in oxic [¹³C]-cellulose treatments (Table 24). Although soil fungi are major degraders of cellulosic biomass under oxic conditions (de Boer et al. 2005; Lynd et al. 2002) no fungal species was labeled by [¹³C]-cellulose, neither under oxic nor under anoxic conditions. The lack of fungi under anoxic conditions is consistent with the previous findings that anaerobic cellulolytic fungi are well-known from the intestine of ruminant and non-ruminant herbivores, but not from soil (Gordon and Phillips 1998; Lynd et al. 2002; Teunissen and DenCamp 1993), and further indicates that fungi played no role under anoxic conditions. Under oxic conditions, a niche differentiation of cellulolytic fungi and cellulolytic bacteria, which is governed by a set of variable factors, e.g., accessibility of cellulose fibres, excretion of antifungal compounds by bacteria, or pH, is likely (Bastian et al. 2009; de Boer et al. 2005; Lynd et al. 2002). Plant material with low lignin content may be more readily accessed by cellulolytic bacteria, and aerobic cellulolytic bacteria may be more competitive at neutral pH and high moisture contents (de Boer et al. 2005; Hiroki and Watanabe 1996). However, the growth of fungi was observed in microcosms with wet soil and cellulosic paper sheets (2.3.3.3) which indicates that fungi were active if the experimental conditions are suitable. Well-known fungi that contribute to the degradation of cellulosic material in aerated soil are related to the phyla *Ascomycota* (e.g., *Aspergillus*, *Fusarium*, *Trichoderma*), *Basidiomycota* (e.g., *Agaricus*, *Cryptococcus*, *Phanerochaete*), and *Mucoromycotina* (e.g., *Mucor*, *Rhizopus*) (Bastian et al. 2009; Eriksson 1978; Hammel 1997; Herculano et al. 2011; Lynd et al. 2002; Mandels and Reese 1999; Mahmood et al. 2006). It is likely that soil fungi were participants in the consumption of cellulose in this study, but the experimental conditions in stable isotope probing experiments (i.e., the near neutral pH, the purity of the cellulose, high moisture content, and constant shaking of slurries) have favored cellulolytic bacteria both under oxic and anoxic. Further research is needed to resolve the role of fungi for the degradation of plant-derived saccharides in the investigated soil.

In contrast to fungal species, protists were labeled in oxic cellulose treatments (i.e., *Eustigmataceae*, *Bodonidae*, *Chrysophyceae*, *Opisthnectidae*, *Mallomonadaceae*, *Stramenopiles*; Table 24), but none of the detected taxa has been described as being cellulolytic (Berner and Berner 1993; Hall 1953; Laybourn-Parry and Parry 2000). Cellulolytic protists are frequently detected in gut ecosystems of ruminants and wood-feeding termites in which they catalyze the breakdown of cellulosic plant material (Bohatier et al. 1990; Breznak and Brune 1994; Coleman 1984; Hidayat et al. 1993; Wheeler et al. 2007). For example, *Ophryoscolecidae*-related ciliates are important anaerobic degrader of cellulose in the rumen of sheep (Coleman 1978) and important constituents of the cellulolytic eukaryotic community in cattle (Kudo et al. 1990). Taxa detected in the current study (Table 24) did not affiliate with species of cellulolytic protists known from ruminal ecosystems what indicates that the primary oxic nature of the soil did not favor anaerobic protists. *Opisthnecta*-related species are aerobic heterotrophic free-living ciliates that occupy ephemeral water bodies or moisture films around soil particles (Bochdansky and Huang 2010; Laybourn-Parry and Parry 2000; Williams and Clamp 2007). Members of this genus are bacteriovorous and often found in nutrient rich wastewaters (Ettl 2001; Foissner 1978). *Bodonidae* are to the most frequently detected heterotrophic flagellates in several ecosystems (Patterson and Lee 2000; Scheckenbach et al. 2006). Members of these genera are frequently associated with aggregates of organic matter, e.g., in activated sludge, on which they graze very effectively on attached bacteria (Laybourn-Parry and Parry 2000; von der Heyden and Cavalier-Smith 2005). Some species, e.g., members of the genus *Bodo*, are able to survive also at low concentrations of prey and can therefore outcompete other bacteriovorous protists that need higher bacterial densities for multiplication (Laybourn-Parry and Parry 2000; Sleight 2000; Zubkov 1995). Similar, *Chrysophyceae*-related flagellates prefer bacteria as energy and carbon source (Caron et al. 1986; Yubuki et al. 2008). *Chrysophyceae* are important constituents of the marine plankton (Throm 1997), but can also be found in soil samples of different geographical regions (Boenigk et al. 2005). They display an aerobic heterotrophic as well as an autotrophic lifestyle or are mixotrophs, i.e., they combine photoautotrophy with heterotrophy (Sleight 2000). Members of *Chrysophyceae* and other protists can be involved in the composting of rice straw and orchard grass, but their role in the degradation process seems to be of minor importance (Cahyani et al. 2004; Lee et al. 2011). The taxa *Eustigmataceae* (algal stramenopiles) and *Leukarachnion* (amoeboid stramenopiles) occur in marine and freshwater habitats as well as in soils (Andersen 2004; Grant et al. 2009; Hoek et al. 1995), and include photoautotrophs, and heterotrophs that are osmotrophic (Berner and Berner 1993; Grant et al. 2009). Some stramenopiles are phagotrophic bacterivorous, i.e., they feed on living and dead bacteria (Laybourn-Parry and Parry 2000; Sleight 2000). Members of the detected eukaryotic families require moist zones in soil, but can also form cysts during periods of dryness or nutrient limitation (Hall 1953; Findenig et al. 2010; Laybourn-Parry and Parry 2000). In water-saturated habitats phototrophic microeukaryotes are important primary producers of biomass and drive the carbon flow through marine and freshwater ecosystems (Hall 1953; Throm 1997). Most soil flagellates feed on bacteria or assimilate organic compounds (Ekelund and Rönn 1994; Murase et al. 2006; Rönn et al. 2002), but their role for the degradation of cellulosic material is largely unresolved. It is likely that heterotrophic protists incorporated [¹³C]-carbon in the experiments during the ingestion of [¹³C]-labeled saccharide-degrading bacteria.

4.5. Pesticides have Minor Effects on the Metabolism of Cellulose-Degrading Communities

Soil microorganisms in agricultural soils may not only be grazed by bacteriovorous protists, but also impaired by toxicity of pesticides, such as herbicides, fungicides, insecticides or break down products thereof (Chowdhury et al. 2008; Gevaio et al. 2000; Katayama and Kuwatsuka 1991; Katayama et al. 1992; Wainwright 1978). The extensive application of pesticides might alter cellulose-dependent carbon flow through the microbial community. Thus, saccharolytic and cellulolytic taxa that were identified as key organisms of the degradation of cellulose and its breakdown products (2.3.3.3) were analysed for their metabolic response on presence and absence of pesticides (Table 28).

The fungicides Metalaxyl and Chlorothalonil did apparently not affect the hydrolysis of cellobiose and subsequent consumption of glucose at concentrations 10 – 33fold above the concentrations recommended for agriculture use (Figure 24; 2.3.3.1). Although Metalaxyl may inhibit β -glucosidase activity in soil (Monkiedje and Spiteller 2002; Sukul 2006), and Chlorothalonil reduces cellulose degradation in aerated soils (Katayama and Kuwatsuka 1991; Katayama et al. 1992; Suyama et al. 1993b; Suyama et al. 1993a), the saccharolytic community of the investigated soil is likely insensitive to both fungicides or negative effects are not reflected in changed processes (Figure 24). Metalaxyl and Chlorothalonil can be decomposed by microorganisms (Fogg et al. 2003; Monkiedje et al. 2003), but it is unlikely that they were degraded during the short incubation time (2 – 4 days) of the experiments of this work (2.3.3.1). Half-life times of Metalaxal and Chlorothalonil are 9 to 66 days (Table 5). Hence, the fungicides likely can interact with the microbes in soil slurries. Several reasons that account for the lack of inhibition at such 'high' concentrations of pesticides are conceivable: (i) Pesticide-sensitive saccharide-degrading fungi were not favoured under the experimental conditions and bacteria were pesticide-insensitive. (ii) Sorption of Metalaxyl and Chlorothalonil to soil particles lowered their biotoxicity due to reduced interactions with soil microorganisms (Fogg et al. 2003; Katayama et al. 1991; Sigler et al. 2003). (iii) Parts of the saccharide-degrading community were selectively inhibited by the pesticides, but other functionally redundant and pesticide-insensitive taxa mimicked this effect. However, it remains unclear if and how both fungicides impacted the saccharide-degrading community, but the effects of Metalaxyl and Chlorothalonil on the degradation of saccharides seem to be minimal. In contrast, the pesticides Bentazon, MCPA and Nonylphenol reduced the degradation of cellulose and cellulose-derived sugars (Figure 25 – Figure 27, Table 28; 3.5).

Aerobic and anaerobic hydrolysis of cellobiose was apparently not reduced by low concentrations of Bentazon, MCPA and Nonylphenol (Figure 25, Table 27), suggesting that these pesticides have minimal effects on the activity of β -glucosidases or endoglucanases (Figure 2) at *in situ*-relevant concentrations. MCPA that is applied at recommended dose does not affect either carbon dioxide production, or O₂ uptake and N-mineralization in a cropland soil, and aerobic cellulose degradation is only little reduced even when MCPA is directly spread on cellulose sheets (Grossbard 1971; Schröder 1979). Although Nonylphenol can also inhibit cell growth of *Bacteria* in pure culture and reduces the nitrifying-activity in activated sludge (Dokianakis et al. 2006; Okai et al. 2000a), it has no impact on cellulolytic members of the bacterial soil community, e.g., *Cytophaga*-related species (Chang et al. 2007).

In accordance with previous studies (Cernakova et al. 1991; Hseu 2006; Marsh et al. 1978; Piutti et al. 2002), the toxic effect of Bentazon, MCPA, and Nonylphenol on the degradation of cellulose-derived carbon, and linked processes was dose-dependent and only evident at concentrations far above values that are typical in pesticide-treated cropland soils (Figure 26, Table 27). The degradation of crystalline cellulose was impaired both under oxic and anoxic conditions, i.e. Bentazon, MCPA, and Nonylphenol reduced the amount of degraded cellulose by about 40 – 60% under oxic conditions and by more than 90% under anoxic conditions (Figure 27, Table 27), indicating that the anaerobic cellulolytic community was highly sensitive to the toxic effects of the pesticides. A similar degree of inhibition has been reported for Oxyfluorfen, Picloram, Metolachlor, and Alachlor under oxic conditions (Kucharski and Wyszowska 2008; Sahid and Yap 1994; Sahid and Ramil 1995). Application of these pesticides may decrease cellulase activity and cell numbers of cellulolytic bacteria (Sahid and Yap 1994; Sahid and Ramil 1995). Sulfosulfuron inhibits the growth of cellulolytic bacteria and other functional groups even at concentrations that are typical of field use (Kucharski and Wyszowska 2008). Similar to cellulose-supplemented treatments (Figure 27) anaerobic processes, especially the consumption of glucose, were strongly inhibited by all pesticides at high concentrations (Figure 26, Table 27). This is in congruence with studies that showed that Bentazon can lower the total microbial biomass after application to soil (Piutti et al. 2002), and can reduce nitrogen mineralization, dinitrogen fixation, soil respiration, and the cultivability of cellulolytic soil bacteria, cellulolytic fungi and *Actinomycetes* (Ahtainen et al. 2003; Marsh et al. 1978, Cernakova et al. 1991).

A generally increased sensitivity of anaerobes is likely, since some fungicides may reduce cellulose decomposition and may impair cellulolytic *Clostridia* as it has been shown for a flooded soil (Katayama and Kuwatsuka 1991). This is consistent with the lower production of carbon dioxide and molecular hydrogen in anaerobic pesticide-supplemented treatments, and the reduction of ferric iron to levels similar to the control without pesticide (Figure 27, Table 27). It is conceivable that Bentazon, MCPA, and Nonylphenol impacted on membrane- and cytosol-associated proteins, and thus did not only specifically inhibit the activity of cellulolytic and saccharolytic taxa, but also the activity of anaerobes (e.g., iron reducers) that may consume fermentation products of anaerobic cellulose degradation, i.e., organic acids and alcohols. This conclusion correlates with reduced transcript numbers of total soil *Bacteria* and saccharide-degrading taxa at the end of the experiment (Figure 28, Table 28). It can be speculated that impairment of anaerobic processes in well-aerated soils is of minor importance for the degradation of cellulose since this process is mainly catalyzed by aerobic organisms in oxic zones. In permanently anoxic soil, e.g., flooded rice fields, pesticides may have stronger impacts on the active microbial community and may significantly inhibit the cellulose-linked carbon flow.

Development of primers that are specific for Cluster III *Clostridiaceae* failed (Table 26, 3.4), but Cluster I *Clostridiaceae* were inhibited by pesticides. A major proportion of known anaerobic cellulolytic bacteria that have been isolated belong to Cluster III *Clostridiaceae*. Thus, it cannot be excluded that members of Cluster III *Clostridiaceae* were also impaired and contributed to the effect of reduced degradation of crystalline cellulose. However, the saccharolytic taxon *Micrococcaceae* and the cellulolytic taxon *Cellulomonadaceae* were apparently not influenced by the application of pesticides since their RNA content was not lowered at the end of the incubation (Table 28). *Actinobacteria* are well known for their

insensitivity to xenobiotics and their potential to degrade a wide range of pesticides, e.g., benzonitrile herbicides and para-nitrophenol (Chikere et al. 2009; Nielsen et al. 2011; Vesela et al. 2010), and thus, may have contributed to the degradation of cellulose despite the presence of pesticides. The capability of some *Bacteria* to degrade xenobiotics (e.g., Ekelund et al. 1993; Hseu 2006; Porter and Hayden 2002) is further reflected in the rapid decrease of Nonylphenol under oxic conditions (Figure 27). Although no degradation products of Nonylphenol were detected, the production of carbon dioxide was less reduced in Nonylphenol-supplemented treatments than in the other pesticide-supplemented treatments (Figure 27, Table 27). This suggests that carbon dioxide that results from the degradation of Nonylphenol masked the impact of Nonylphenol on carbon dioxide production that results from degradation of cellulose. Interestingly, growth of fungi on cellulose sheets was observed (data not shown) that suggests that cellulolytic fungi were insensitive to the pesticide toxicity, as it was shown in previous studies (Girlanda et al. 2009; Koroleva et al. 2002; Pampulha et al. 2007). However, the impact of pesticides on fungi in the current study remains speculative.

Exposure of the soil organisms to high concentrations of pesticides is possible although when pesticides are applied at recommended rate. Heterogeneous distribution of pesticides in crop fields may lead to local maxima of pesticide concentration in soil that exceed reported mean values (Marsh et al. 1978). Plants can accumulate pesticides in their tissue (Bokern and Harms 1997; Porter and Hayden 2002). Likewise, cellulolytic microorganisms that use these pesticide-treated plants as carbon and energy source can be exposed to higher concentrations as it was originally applied. Nevertheless, current and previous data suggest (i) that the anaerobic saccharide-degrading community is more sensitive to the toxicity of pesticides than the aerobic one and (ii) that the impact of Bentazon, MCPA and Nonylphenol on the saccharide-degrading community is minimal at *in situ*-relevant concentrations (Hypothesis 3, 1.7).

4.6. Conclusions and Model

The results of experiments that were conducted within the doctoral project illustrate the complexity of the cellulose-linked food web. Prokaryotic and eukaryotic taxa that are actively involved in the turnover of carbon in soil slurries of an aerated agricultural soil were identified. Cellulose and cellulose-derived saccharides are utilized by a highly diverse aerobic and anaerobic microbial community, of which a large proportion was represented by new cellulolytic and saccharolytic species (Hypothesis 1, 1.7; Figure 34). Fluctuations of the availability of O₂ did not impact on the capability of soil microbial community to consume supplemented carbon compounds (Hypothesis 2, 1.7; Figure 34).

Agricultural soils are often treated with pesticides, which might have impacts on the aerobic and anaerobic degradation of plant-derived saccharides and the involved microbial community (Hypothesis 3, 1.7; Figure 34). The results of the presented experiments do not support this hypothesis. Tested pesticides did not impact on the degradation of saccharides at *in situ*-relevant concentrations. It is possible that single saccharide-degrading taxa were impacted by pesticides, particular anaerobes, but this effect apparently plays no role for the capacity of the soil microbial community to degrade the supplemented saccharides.

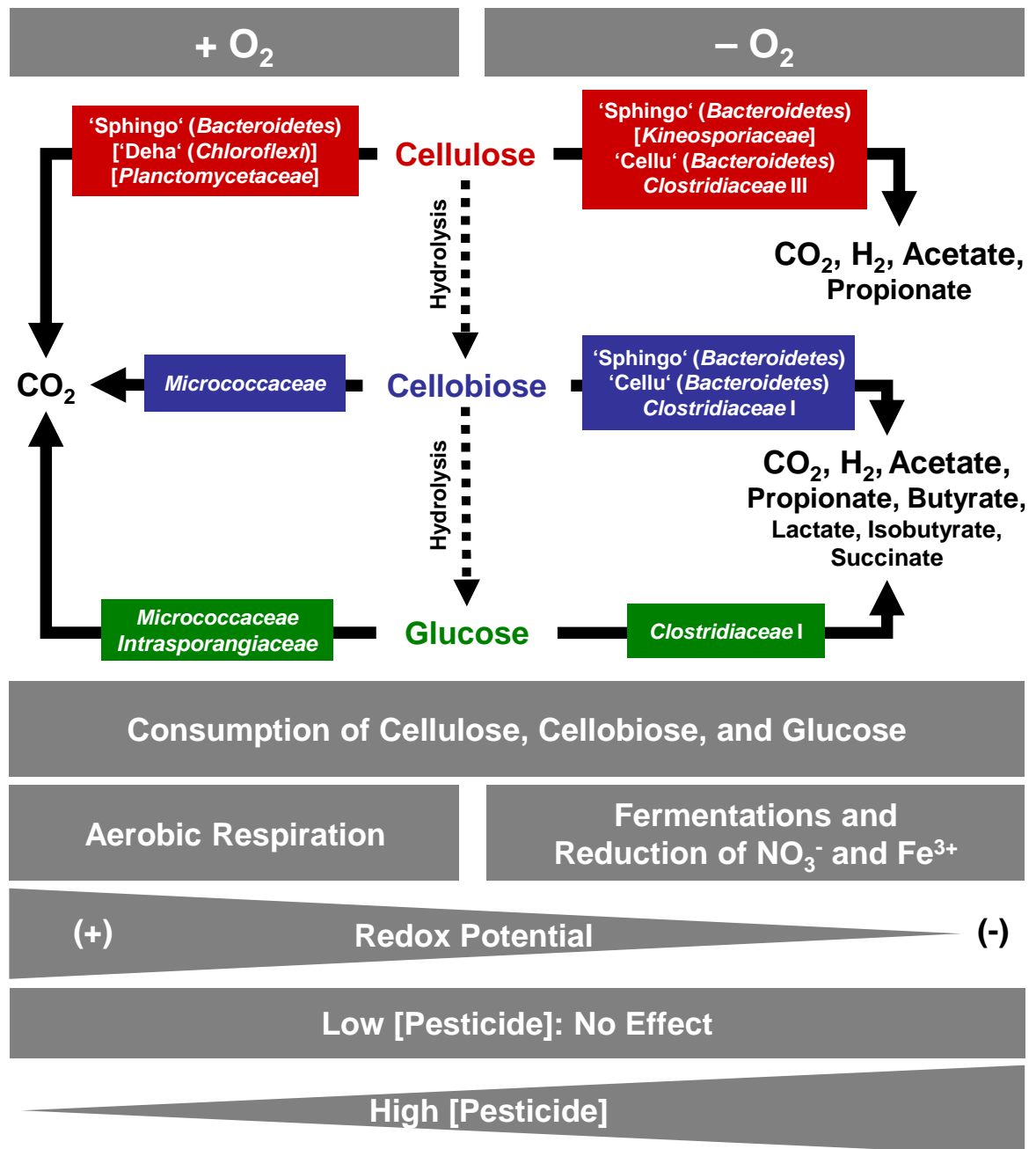


Figure 34. Conceptual model of processes related to the degradation of cellulose, major cellulolytic and saccharolytic bacterial taxa, and the impact of pesticides in the presence and absence of O_2 in an aerated agricultural soil under experimental conditions. The gray shaded boxes in the lower portion of the model illustrate different events that occur during the conversion of saccharides to products. The relative amounts of degradation products are indicated by different font sizes. Proposed names of bacterial taxa in quotation marks represent new family-level taxa without cultured representatives (Table 22). Bacterial taxa in brackets have not been previously shown to be cellulolytic, but were labeled in cellulose-supplemented treatments. The tapered box for redox potential indicates that the redox potential became more negative in the anoxic period. The tapered box for high concentrations of pesticides indicates that high concentrations only inhibited the utilization of saccharides under anoxic conditions.

Agricultural soils are usually well-aerated soils. Nonetheless, oxic and anoxic microzones co-occur (1.6) in which aerobic and anaerobic microbial activities take place (Figure 3). The collective results of aerobic and anaerobic degradation processes show that cellulose, cellobiose, and glucose are mineralized to carbon dioxide by aerobic respiration under oxic conditions and that the cellulose, cellobiose, and glucose are converted to fermentation products, concomitant with the apparent reduction of nitrate and ferric iron under anoxic conditions (Figure 11, Figure 25 – Figure 27, Figure 30). Acetate is a dominant fermentation product during anaerobic degradation, followed by propionate, butyrate and traces of lactate, isobutyrate and succinate. The detected product spectra correlate well with the identification of different aerobic and anaerobic cellulolytic and saccharolytic bacterial taxa and their known metabolic capabilities (Table 22; 4.2, 4.2.2). [¹³C]-cellulose is mainly degraded by Cluster III *Clostridiaceae*, and new family-level taxa within *Bacteroidetes* under anoxic conditions, whereas new family-level taxa of the phyla *Bacteroidetes* and *Chloroflexi* are key degraders under oxic conditions. Active sub-communities in [¹³C]-cellobiose and [¹³C]-glucose treatments differ from cellulose degrading communities, and are dominated by Cluster I *Clostridiaceae* under anoxic conditions, whereas *Intrasporangiaceae* and *Micrococcaceae* are prevalent in oxic treatments. In addition, the new family-level taxa ‘Sphingo1-4’ and ‘Cellu1-3’ were stimulated by the addition of cellobiose during the anoxic period under fluctuating availabilities of O₂ (Figure 32E and G). However, the role of other active taxa, i.e., *Kineosporiaceae*, *Planctomycetaceae*, and the new family-level taxon ‘Deha1’, was not fully resolved. These taxa likely participate in the degradation of plant-derived saccharides in the investigated soil, but it is still unclear at which level they are involved in the cellulose-linked food web.

In summary, Cluster III *Clostridiaceae* and *Bacteroidetes*-related taxa represented the major cellulolytic constituents of the microbial community in slurries of the investigated agricultural soil, whereas Cluster I *Clostridiaceae*, and the actinobacterial families *Micrococcaceae* and *Intrasporangiaceae* were saccharolytic satellite organisms that utilized cellulose-derived hydrolysis products. The rapid response of these microbial taxa altered the redox potential during the degradation of saccharides (Figure 30), but the rate at which plant-derived saccharides are degraded was largely independent of the availability of O₂. Selective activation of functionally redundant taxa that coexist in oxic and anoxic microzones of aerated agricultural soil likely makes the degradation of cellulose a stable function even when environmental factors change rapidly. Pesticides did apparently not impair the activity of the cellulose degrading-community at *in situ*-relevant concentrations of pesticides. The anaerobic bacterial community seemed sensitive to pesticides, but significant effects are only observed at very high concentrations that are unlikely to occur in agricultural soil.

4.7. Limitation of the Applied Methods

RNA stable isotope probing was used as a method to select for bacterial taxa that actively consumed [¹³C]-cellulose and cellulose-derived hydrolysis products, i.e., cellobiose and glucose, in experiments performed with soil from an agricultural ecosystem. Although these saccharides were applied at concentrations greater than maximum concentrations detected in soils (Hill et al. 2008; Medeiros et al. 2006), they represent a compromise between low *in situ* concentrations and concentrations that are needed for detectable incorporation of [¹³C] in the rRNA pool. The choice of

gradient fractions that are representative for labeled and unlabeled RNA was based on the distribution of RNA in the gradient (Figure 13). The 'heavy' fractions are enriched for [^{13}C]-RNA, but it is likely that they contain unlabeled RNA (Manefield et al. 2002a; Manefield et al. 2002b; Radajewski et al. 2003). To ensure that detected taxa of these 'heavy' fractions were isotopically labeled, tRFLP analyses and the construction of gene libraries were additionally applied. It cannot be excluded that the study missed single cellulolytic and saccharolytic soil taxa due to the constrictive type of data analysis. Some tRFs could not be identified and some active taxa might have also been present in non-analysed fractions. Nevertheless, the identification of 48 active family-level taxa and the dominance of well-characterized cellulolytic and saccharolytic microorganisms (e.g., Cluster I and III *Clostridiaceae* or *Micrococcaceae*) in tRFLP profiles and gene libraries (Table 22; 3.2.3.2.1, 3.2.3.2.2) indicates that the majority of the cellulose-degrading community was covered by the applied way of data analysis.

It was possible to monitor community dynamics during the degradation of saccharides (Table 19, Table 20). Although labeling of some taxa at a later time point due to assimilation of [^{13}C]-carbon dioxide cannot be excluded, cross-feeding should have been minimal as the gaseous products were periodically removed and growth of photoautotrophic organisms was prevented by dark incubation. Taxa that were labeled at later time points in cellulose treatments might have needed longer for the competitive utilization of cellulose or might have utilized soluble hydrolysis products from cellulose, e.g., sugars or fatty acids (Bayer et al. 2006). The degradation of [^{13}C]-enriched wheat straw, rice callus, or cellulose by agricultural soil communities also reveals [^{13}C]-labeling of microbial taxa after prolonged incubation of which is not known if they represent cellulolytic taxa (Bernard et al. 2007; Lee et al. 2011; Li et al. 2009). Stable isotope probing can shed light on microbial food webs in various habitats (e.g. Hamberger et al. 2008; Hunger et al. 2011; Murell and Radajewski 2000; Wüst et al. 2009), but cross-feeding complicates interpretation of labeling patterns, especially for newly discovered taxa with unknown metabolic potentials. Therefore, the labeling of known and new cellulolytic and saccharolytic taxa was qualified by taxon-specific qPCR approaches in the current study (3.4).

The degradation of CMC does not necessarily require endoglucanases what makes CMC a substrate that can also be used by saccharolytic organisms (Bisaria and Ghose 1981). For example, *Pantoea* species lack endoglucanase-encoding genes and utilizes CMC, but not crystalline cellulose (Adams et al. 2011). However, the degree of substitution (DS), i.e., the number of hydroxyl groups that are esterified with carboxymethyl groups per glucose unit, of the used CMC was high (i.e., 0.65 - 0.9). The higher the DS of CMC is, the more difficult the substrate is to degrade and the more similar it is to crystalline cellulose (Reese et al. 1950). Therefore, the used form of CMC was a suitable analog for crystalline cellulose. Taking all these considerations into account it can be concluded that the identified cellulolytic and saccharolytic taxa are likely key microorganism for the degradation of cellulose and cellulose-derived carbon in the investigated agricultural soil.

4.8. Future Perspectives

Central objectives of this doctoral thesis were to get detailed insights into the cellulolytic and saccharolytic prokaryotic soil community that facilitates the aerobic and anaerobic degradation of cellulose. It was experimentally figured out that contrasting availabilities of O₂ selectively activate cellulolytic and saccharolytic bacterial taxa, and that these taxa facilitate redox changes. However, it was not analyzed how frequent switches between oxic and anoxic cycles affect active bacteria and the redox potential.

A huge uncultured bacterial diversity was active in the investigated agricultural soil of which metabolic capabilities are unknown. The role of the saccharide-degrading taxa, i.e., *Planctomycetaceae*, *Kineosporiaceae*, for the degradation of plant-derived carbon *in situ* has also not been resolved. It would be intriguing to isolate members of new family-level taxa and to analyse other types of well-aerated soil, e.g., forest soil, grassland or deserts, for the existence of these groups. The dominance of fungi and *Bacteria* as important degraders of cellulosic biomass can shift in aerated forest soil (Dilly et al. 2001), whereas *Bacteria* dominate the decomposition of carbon in anoxic zones of forest leaf litter (Reith et al. 2002). Decomposition of organic material in desert soil can be negligible during long periods of drought, but is immediately starting after rain fall events (Jacobson and Jacobson 1998). Termites, beetle larvae, and fungi dominate cellulose-degrading communities in desert soil, but it is suggested that *Bacteria* also contributes to this process (Jacobson and Jacobson 1998). In this regard, it would be interesting to analyse if the degradation of plant-derived carbon is also a stable community function in such ecosystems and how active groups of saccharide-utilizers respond to shifts in their environment. Likewise, analysis of the archaeal community can clarify if mesophilic *Archaea* are parts of the cellulolytic soil community in non-agricultural temperate soils or if cellulolytic *Archaea* only exist in extreme environments, e.g., in hot springs (Graham et al. 2011; Perevalova et al. 2005).

Active aerobic and anaerobic saccharide-degrading key taxa, e.g., Cluster I *Clostridiaceae*, *Micrococcaceae*, or new family-level taxa of *Bacteroidetes*, were quantified with taxon-specific qPCR assays. It should be possible to use these primers as taxon-specific probes to determine the spatial distribution of these microbial groups in soil and on cellulosic substrates by fluorescence *in situ* hybridization (FISH). The localisation of especially new family-level taxa may reveal if a taxon is directly attached to the surface of cellulosic material or if it is located in the surrounding environment and rather acts as a saccharolytic satellite organism (O'Sullivan et al. 2007).

[¹³C]-cellobiose and -glucose were used in this study to distinguish between truly cellulolytic species and saccharolytic satellite species that solely feed on soluble hydrolysis products. In nature, cellulose is embedded in a lignocellulose complex. The capability to mineralize lignin and lignocellulosic compounds is well-known for many soil fungi, but only few studies have addressed the *in situ* diversity of soil bacteria that are able to degrade lignin (Kirk and Farrell 1987; Tuomela et al. 2000). This study did not address the degradation of lignin or hemicellulose. It can be hypothesized to perform comparative stable isotope probing experiments with more recalcitrant compounds, e.g., [¹³C]-labeled lignin and labeled plant material. This would allow for the identification of species that catalyse the degradation of lignin or lignocellulose, and would shed additional light on the trophic links between organisms that are involved in turnover of plant organic matter in soils.

5. SUMMARY

The polysaccharide cellulose is a major component of organic matter in terrestrial ecosystems and its mineralization drives carbon fluxes in soil. The degradation of plant-derived saccharides (e.g., cellulose, cellobiose, and glucose) is catalysed by a huge diversity of aerobic and anaerobic microorganisms (including *Bacteria*, fungi, and protists), but there is limited information about their phylogenetic identities and their *in situ* relevance in soil. Soil is a heterogeneous habitat in which oxic and anoxic microzones co-occur, and in which the distribution of O₂ can change rapidly. Hence, the availability of O₂ is an important factor that determines the activity of the saccharide-degrading community in microzones of aerated soil. Likewise, the accumulation of potential toxic pesticides in agricultural ecosystems might influence microbial activity. It is not resolved how active cellulolytic and saccharolytic taxa respond to rapid changes in the availabilities of O₂. Furthermore, it is unclear if pesticides impact on the degradation of cellulose and cellulose-linked processes, and influence the activity of active saccharide-utilizing microorganisms.

Hence, this study first identified cellulolytic and saccharolytic aerobic and anaerobic *Prokaryotes* that catalyzed the degradation of supplemented [¹³C]-labeled saccharides by 16S rRNA stable isotope probing. The metabolic response of major bacterial taxa to pesticides and fluctuating availabilities of O₂ was further analyzed with taxon-specific qPCR assays. *Eukaryotes* that contributed to soil carbon flux were identified by targeting 18S rRNA genes by the collaborative group of Dr. A. Chatzinotas at the Helmholtz Centre (UFZ) in Leipzig.

Cellulose, cellobiose, and glucose were mineralized to carbon dioxide under oxic conditions, whereas different fermentation products accumulated under anoxic conditions. Fermentations occurred concomitant with the apparent reduction of nitrate and ferric iron. The degradation of supplemented saccharides was stable under oxic and anoxic conditions. *Archaea* were no active constituents of the cellulose-degrading community in the investigated soil. [¹³C]-cellulose was mainly degraded by *Kineosporiaceae* (*Actinobacteria*), the cellulolytic taxon Cluster III *Clostridiaceae* (*Firmicutes*), and the new family-level taxon 'Cellu1-3' (*Bacteroidetes*) under anoxic conditions. Under oxic conditions, the new family-level taxa 'Sphingo1-4' (*Bacteroidetes*) and 'Deha1' (*Chloroflexi*), and *Planctomycetaceae* (*Planctomycetes*) were involved. Active community members in [¹³C]-cellobiose and [¹³C]-glucose treatments differed from those in [¹³C]-cellulose treatments, and were selectively activated by O₂. Twenty-eight of the 48 labeled bacterial family-level taxa did not closely affiliate with cultured species.

Labeled *Eukaryotes* belonged to the families *Bodonidae*, *Eustigmataceae*, *Mallomonadaceae*, *Opisthokontidae*, unclassified *Chrysophyceae*, and unclassified *Stramenopiles*. These families inhabit autotrophic algae and bacteriovorous flagellates. It is likely that these active *Eukaryotes* were labeled by incorporation of [¹³C]-carbon derived from grazing on active cellulolytic and saccharolytic soil bacteria. Fungi were not labeled in stable isotope probing experiments.

The pesticides Bentazon, MCPA and Nonylphenol impaired cellulose-linked microbial processes only at pesticide concentrations far above the recommended rate. The impairment was most pronounced under anoxic conditions. *Planctomycetaceae* and the new family-level taxon 'Sphingo1-4' were sensitive to pesticide addition under oxic conditions, whereas Cluster I *Clostridiaceae* and the new family-level taxon 'Cellu1-3' were reduced under anoxic conditions.

Nevertheless, the impact of pesticides on the degradation of saccharides at *in situ*-relevant concentrations seems to be minimal.

These collective findings suggest that (i) a large uncultured diversity of *Bacteria* was involved in the degradation of cellulose, (ii) O₂ selectively activates different cellulolytic and saccharolytic taxa, (iii) Cluster III *Clostridiaceae*, and the new family-level taxa 'Sphingo1-4' and 'Cellu1-3' represent the major cellulolytic constituents of the microbial community in the investigated agricultural soil, whereas Cluster I *Clostridiaceae*, *Intrasporangiaceae* and *Micrococcaceae* are saccharolytic satellite organisms that utilize cellulose-derived carbon, and (iv) the degradation of plant-derived saccharides is a community function that is stabilized by the rapid response of active bacterial taxa and independent of fluctuating availabilities of O₂ and of pesticide application.

6. ZUSAMMENFASSUNG

Das Polysaccharid Zellulose stellt in terrestrischen Ökosystemen einen Hauptbestandteil organischer Substanz dar und treibt durch seine Mineralisierung den Kohlenstofffluss in Böden an. Der Abbau von pflanzlichen Zuckern (z.B. Zellulose, Zellobiose und Glukose) wird von einer großen Diversität aerober und anaerober Mikroorganismen (Bakterien, Pilze und Protisten eingeschlossen) katalysiert, allerdings ist das Wissen über deren phylogenetische Zuordnung, sowie deren ökologische Relevanz in Böden begrenzt. Böden selbst stellen einen heterogenen Lebensraum dar, in dem oxische und anoxische Mikrozonen nebeneinander auftreten und sich die Verteilung von Sauerstoff (O₂) schnell ändern kann. In gut belüfteten Böden kann die Verfügbarkeit von Sauerstoff deshalb als ein ausschlaggebender Faktor für die Aktivität der zuckerabbauenden mikrobiellen Gemeinschaft gesehen werden. Des Weiteren ist es denkbar, dass die Akkumulation von potenziell giftigen Pestiziden einen Einfluss auf die mikrobielle Gemeinschaft ausübt. Es ist nicht geklärt, in wie fern aktive zellulolytische (d.h. zelluloseabbauende) und saccharolytische (d.h. zellobiose- und glukoseabbauende) Gruppen auf plötzliche Änderungen der Sauerstoffverfügbarkeit reagieren. Ebenso ist es weitgehend unbekannt, ob Pestizide den Abbau von Zellulose und die Aktivität von aktiven zuckerabbauenden Mikroorganismen beeinträchtigen.

Die vorliegende Studie identifizierte deshalb mit Hilfe von 16S rRNA basierter Stabiler-Isotopenbeprobung aerobe und anaerobe Prokaryoten, die den Abbau von zugegebenen [¹³C]-markierten Zuckern katalysierten. Die metabolische Reaktion bedeutender bakterieller Gruppen auf Pestizide sowie wechselnde Sauerstoffverfügbarkeit wurde für mit Hilfe von gruppen-spezifischen qPCR-Assays untersucht. Am Kohlenstofffluss beteiligte Eukaryoten wurden auf Basis ihrer 18S rRNA Gene durch die Arbeitsgruppe von Dr. A. Chatzinotas am Umweltforschungszentrum Leipzig (UFZ) identifiziert.

Zellulose, Zellobiose sowie Glukose wurden unter oxischen Bedingungen vollständig zu Kohlenstoffdioxid abgebaut, wohingegen verschiedene Gärungsprodukte während des anaeroben Abbaus akkumulierten. Gärungsprozesse liefen dabei zeitgleich mit der Reduktion von Nitrat und Eisen ab. Der Abbau zugesetzter Zucker verlief sowohl unter oxischen, als auch unter anoxischen Bedingungen stabil. Archaeen stellten im untersuchten Boden keinen aktiven Bestandteil der zuckerabbauenden Gemeinschaft dar. [¹³C]-markierte Zellulose wurde unter anoxischen Bedingungen vornehmlich von Vertretern der *Kineosporiaceae* (*Actinobacteria*), zellulolytischen Vertretern des Cluster III der *Clostridiaceae* (*Firmicutes*), sowie der neu definierten Familien-ähnlichen Gruppe „Cellu1-3“ (*Bacteroidetes*) abgebaut. Unter oxischen Bedingungen waren die neuen Familien-ähnlichen Gruppen „Sphingo1-4“ (*Bacteroidetes*) und „Deha1“ (*Chloroflexi*), sowie Vertreter der *Planctomycetaceae* (*Planctomycetes*) die beteiligten Hauptgruppen. Die aktive [¹³C]-zellobiose- und [¹³C]-glukoseabbauende Gemeinschaft unterschied sich von der [¹³C]-zelluloseabbauenden Gemeinschaft. Des Weiteren fand eine selektive Aktivierung aktiver aerober und anaerober Gruppen durch das Vorhandensein bzw. Fehlen von Sauerstoff statt. 28 der 48 detektierten und markierten bakteriellen Familien wiesen keinen bekannten kultivierten Vertreter auf und stellten somit neue Familien-ähnliche Gruppen dar.

Markierte Eukaryoten wurden den Familien *Bodonidae*, *Eustigmataceae*, *Mallomonadaceae*, *Opisthokontidae* sowie nicht klassifizierten *Chrysophyceae* und *Stramenopiles* zugeordnet. Diese Familien werden von autotrophen Algen und bakterivoren Flagellaten repräsentiert. Es ist sehr wahrscheinlich, dass die [¹³C]-Markierung dieser Eukaryoten aus der Beweidung von aktiven zellulolytischen und saccharolytischen Bodenbakterien resultiert. Pilze wurden in keinem Experiment der Stablen-Isotopenbeprobung markiert.

Die Pestizide Bentazon, MCPA sowie Nonylphenol beeinträchtigten den Zelluloseabbau und assoziierte mikrobielle Prozesse ausschließlich unter sehr hohen Pestizidkonzentrationen, die die empfohlene Dosis um ein Vielfaches überschritten. Anaerobe Prozesse wurden dabei am Stärksten beeinträchtigt. Des Weiteren sank unter oxischen Bedingungen die Abundanz der Familie *Planctomycetaceae* und die der neuen Familien-ähnlichen Gruppe „Sphingo1-4“, wohingegen Vertreter des Cluster I der *Clostridiaceae* und die neue Familien-ähnliche Gruppe „Cellu1-3“ unter anoxischen Bedingungen reduziert wurden. Dennoch scheinen *in situ*-relevante Pestizidkonzentrationen nur einen minimalen Effekt auf den Abbau von Zuckern zu haben.

Die gesammelten Ergebnisse zeigen, dass (i) eine große unkultivierte, bakterielle Diversität am Abbau von Zellulose beteiligt ist, (ii) die Verfügbarkeit von Sauerstoff ein ausschlaggebender Faktor für die Aktivierung verschiedener zellulolytischer und saccharolytischer Gruppen ist, (iii) Vertreter des Cluster III der *Clostridiaceae* sowie der neuen Familien-ähnlichen Gruppen „Sphingo1-4“ und „Cellu1-3“ den zellulolytischen Hauptbestandteil der mikrobiellen Gemeinschaft darstellen, wohingegen Vertreter des Cluster I der *Clostridiaceae*, der *Intrasporangiaceae* und der *Micrococcaceae* als saccharolytische Satelliten bezeichnet werden können, da diese freiwerdende Zucker aus Zellulose abbauen und (iv) der Abbau von pflanzlichen Zuckern durch die schnelle metabolische Antwort aktiver bakterieller Gruppen stabilisiert wird und unabhängig von schwankender Sauerstoffverfügbarkeit oder Pestizidapplikation ist.

7. REFERENCES

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8. ACKNOWLEDGEMENTS

I want to thank everyone that has directly and indirectly accounted for the successful preparation of this work.

In particular, Prof. Harold L. Drake for making it possible for me to work on this interesting project, for inspiring discussions, and spending time on me whenever I had problems. Special thanks also go to PD Dr. Steffen Kolb for the great scientific support during this work and helpful comments throughout the last years.

I want to thank all members of the Department of Ecological Microbiology for the convenient and relaxed working atmosphere, and the helpful support whenever I had questions. I am also grateful to Gerhard Kűfner, Peter Schmidt and colleagues for assistance in the planning and construction of incubation chambers.

Best thanks also go to the Deutsche Forschungsgemeinschaft and the University of Bayreuth (in particular Robert Debusmann & woman's officer) for financial support.

I want to thank Dr. Antonis Chatzinotas and his working group of the Umweltforschungszentrum Leipzig for analyzing the eukaryotic diversity. I also thank Stephan Schulz, Prof. Dr. Michael Schloter and Prof. Dr. Jean Charles Munch of the Technical University Munich for providing soil samples.

Special thanks further go to Pia Wűst, Sindy Hunger, Daniela Degelmann and Christina Hirsch for technical assistance, proofreading, lots of nice coffee parties and for listening to and resolving many of my (scientific) problems.

I also want to thank all my friends, especially Biggi Hauffe, for mental support throughout the work, amazing postcards, and for bringing me back into reality after long nights of laboratory work. I also thank all the people I forgot to mention.

The absolutely greatest thanks go to my friend Chris, my parents and my brother. I want to thank you all for the years of support, for keeping me grounded, and for giving me all the understanding I needed.

9. PUBLICATIONS AND PRESENTATIONS

To date, the results of this work have yielded two publications and one additional manuscript that has been submitted. A fourth manuscript is in preparation. In addition, the work has been presented at numerous national and international conferences.

9.1. Publications and Manuscripts

Parts of this work were published in peer-reviewed Journals:

Schellenberger, S., S. Kolb, and H.L. Drake. 2010. Metabolic responses of novel cellulolytic and saccharolytic agricultural soil *Bacteria* to oxygen. *Environmental Microbiology* **12**: 845-861.

Schellenberger, S., S. Kolb, and H.L. Drake. 2011. Functionally redundant cellobiose-degrading soil *Bacteria* respond differentially to oxygen. *Applied and Environmental Microbiology* **77**: 6043-6048.

Schellenberger, S., S. Kolb, and H.L. Drake. 2011. Impairment of cellulose- and cellobiose-degrading soil *Bacteria* by two acidic herbicides, submitted to FEMS Letters, 2011.

Chatzinotas, A., S. Schellenberger, V. Jaschick, H. L. Drake, and S. Kolb. 2011. Soil protists feed on cellulolytic and saccharolytic soil *Bacteria*, in preparation.

9.2. Presentations with Abstracts

Schellenberger, S., S. Kolb, and H.L. Drake. 2010. Metabolic responses of novel cellulolytic and saccharolytic soil *Bacteria* to oxygen. *BioSpektrum Tagungsband zur VAAM-Jahrestagung: Special Issue 2010*: 214. ISSN 0947-0867.

S. Schellenberger, A. Chatzinotas, V. Jaschick, D. Schlosser, H. L. Drake, and S. Kolb. 2011. Agricultural Soil Protists Assimilate Carbon from Supplemental ¹³C-Cellulose. *BioSpektrum Tagungsband zur VAAM-Jahrestagung: Special Issue 2011*: 119. ISSN 0947-0867.

Schellenberger, S., S. Kolb, and H.L. Drake. 2011. Response of cellulose- and cellobiose-degrading soil *Bacteria* to different redox potentials in an aerated soil. *BioSpektrum Tagungsband zur VAAM-Jahrestagung: Special Issue 2011*: 88. ISSN 0947-0867.

Schellenberger, S., S. Kolb, and H.L. Drake. 2010. Impact of oxygen and herbicides on cellulolytic and saccharolytic agricultural soil bacteria. 13. Internationales Symposium of Microbial Ecology (ISME-13), Seattle, USA, 2010. PS.01.070; Abstract on disc.

From Diplom Studies

Schellenberger, S., S. Kolb, and H.L. Drake. 2008. Atmospheric methane uptake in different forest soils is driven by similar methanotrophic communities. *BioSpektrum Tagungsband zur VAAM-Jahrestagung: Special Issue 2008*: 128. ISSN 0947-0867.

9.3. Presentations without Abstracts

Schellenberger, S., S. Kolb, and H.L. Drake. 2008. Impacts of pesticides and oxygen on microbial cellulose degradation in aerated agricultural soils. 1. Statuscolloquium of SPP 1315 'Biogeochemical Interfaces in Soil'. UFZ Leipzig, Germany, 2008.

Schellenberger, S., S. Kolb, and H.L. Drake. 2009. Impact of pesticides and oxygen on cellulose degradation by prokaryotes in aerated agricultural soils. 2. Statuscolloquium of SPP 1315 'Biogeochemical Interfaces in Soil', Altes Schloß Dornburg, Jena, Germany, 2009.

Schellenberger, S., S. Kolb, and H.L. Drake. 2009. Microbial soil communities involved in cellulose degradation under oxic and anoxic conditions. Workshop 'Molecular Ecology Methods', Julius Kühn-Institut Braunschweig, Germany, 2009.

10. DECLARATION

I declare that this document has been composed by myself, and describes my own work, unless otherwise acknowledged in the text.

This dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution. Material from the published or unpublished work of others, which is referred to in the dissertation as well as all sources of information are acknowledged.

Stefanie Schellenberger

Bayreuth, 11.11.2011

APPENDICES

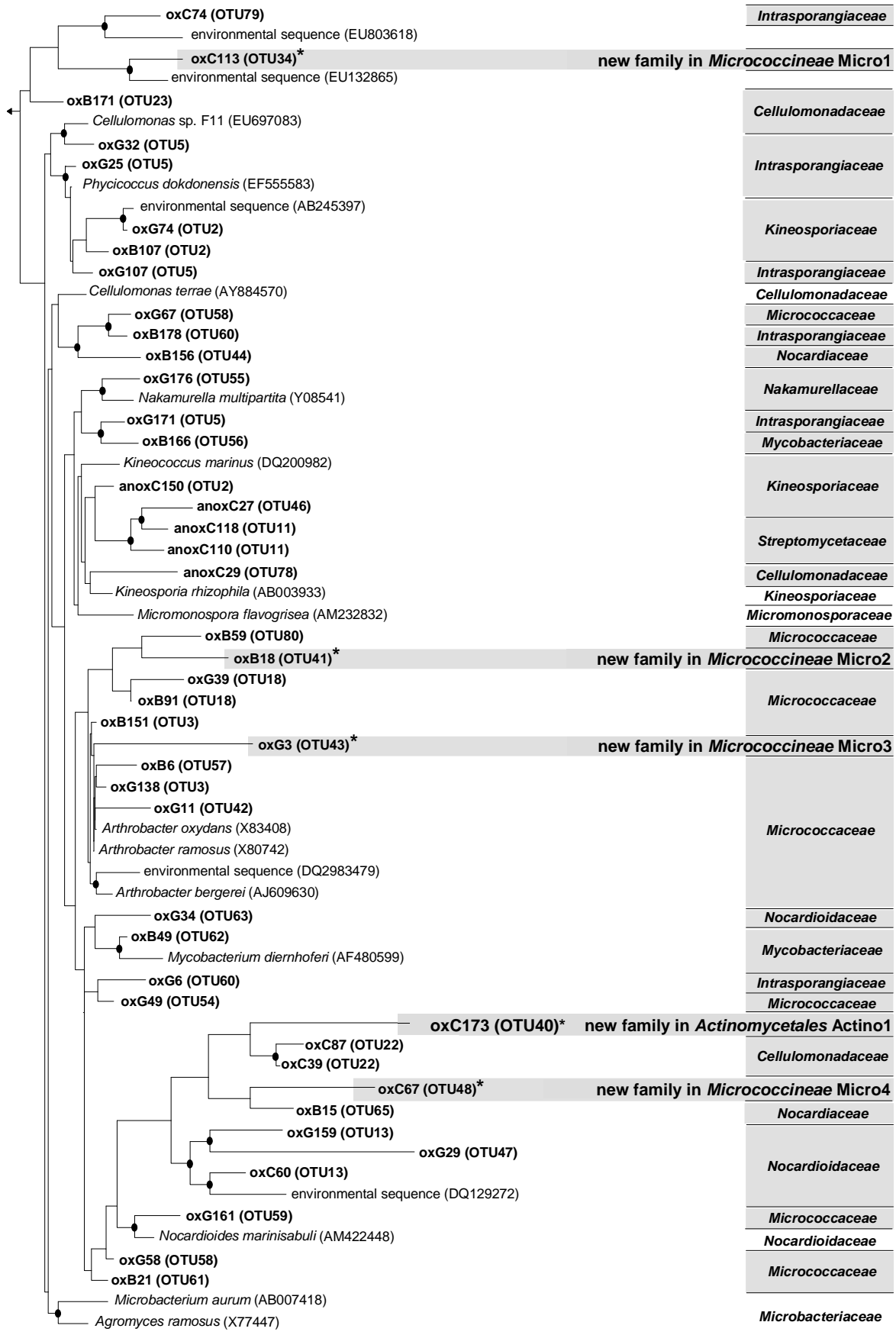
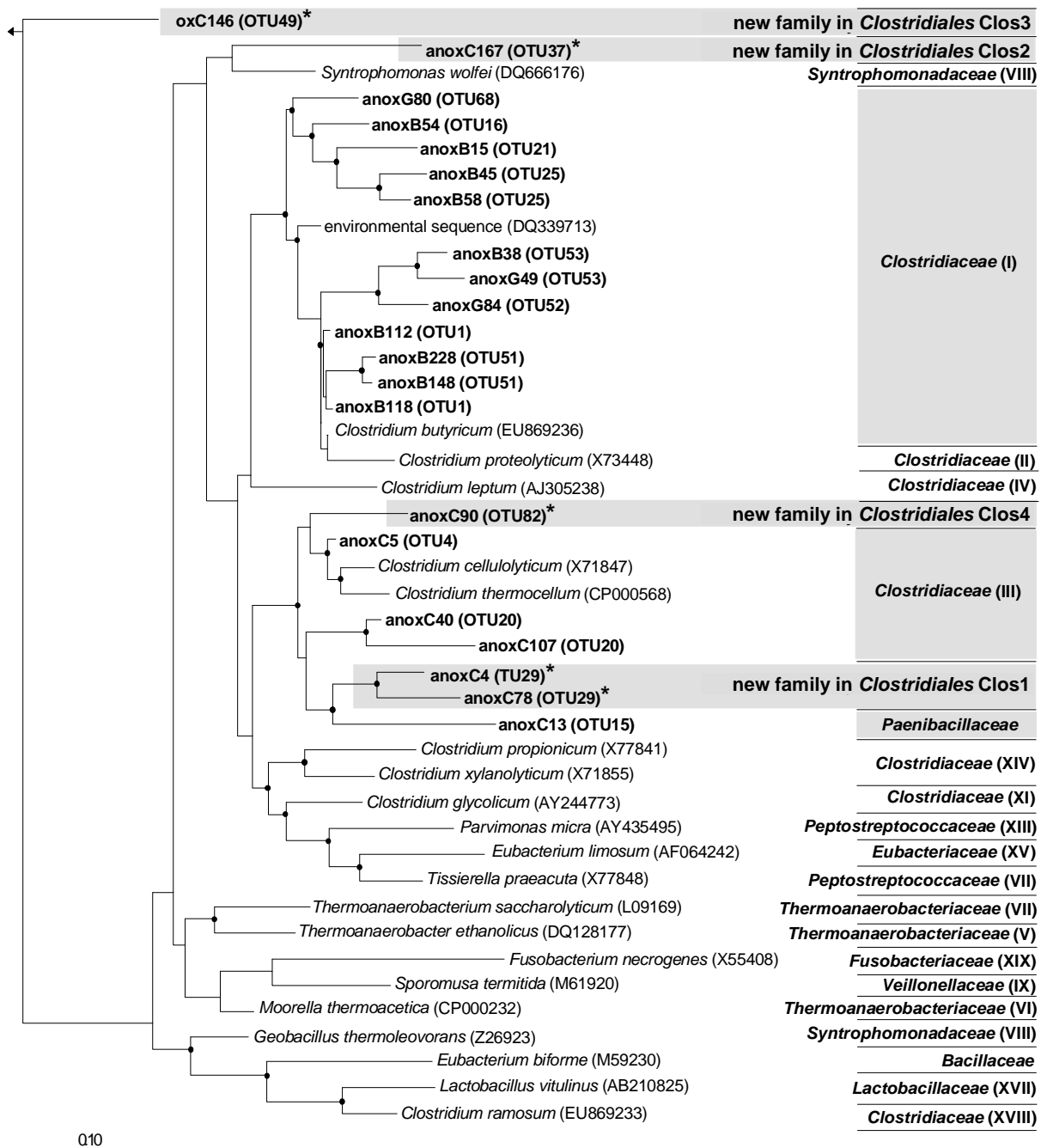


Figure A1. Phylogenetic tree of 16S rRNA cDNA sequences (bold) and reference sequences of the phylum *Actinobacteria*. Accession numbers are in parentheses, and OTU designations are given in Table 22. Shaded areas highlight labeled taxa. Sequences indicative of new families are marked with an asterisk (*). The tree was calculated with AXML (50% filter; 592 valid positions between positions 101 and 727 of the 16S rRNA gene of *Escherichia coli*). Dots at nodes indicate confirmation of topology by neighbor joining and parsimony using the same dataset. The out group was *Methanosarcina barkeri* (AF028692). Scale bar, 10% evolutionary distance. Abbreviations: ox, oxic microcosm; anox, anoxic microcosm; C, cellulose; B, cellobiose; G, glucose.



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Figure A2. Phylogenetic tree of 16S rRNA cDNA sequences (bold) and reference sequences of the phylum *Firmicutes*. Accession numbers are in parentheses, and OTU designations are given in Table 22. Shaded areas highlight labeled taxa. Sequences indicative of new families are marked with an asterisk (*). The tree was calculated with AXML (50% filter; 623 valid positions between positions 136 and 830 of the 16S rRNA gene of *Escherichia coli*). Dots at nodes indicate confirmation of topology by neighbor joining and parsimony using the same dataset. The out group was *Methanosarcina barkeri* (AF028692). Scale bar, 10% evolutionary distance. Abbreviations: ox, oxic microcosm; anox, anoxic microcosm; C, cellulose; B, cellobiose; G, glucose.

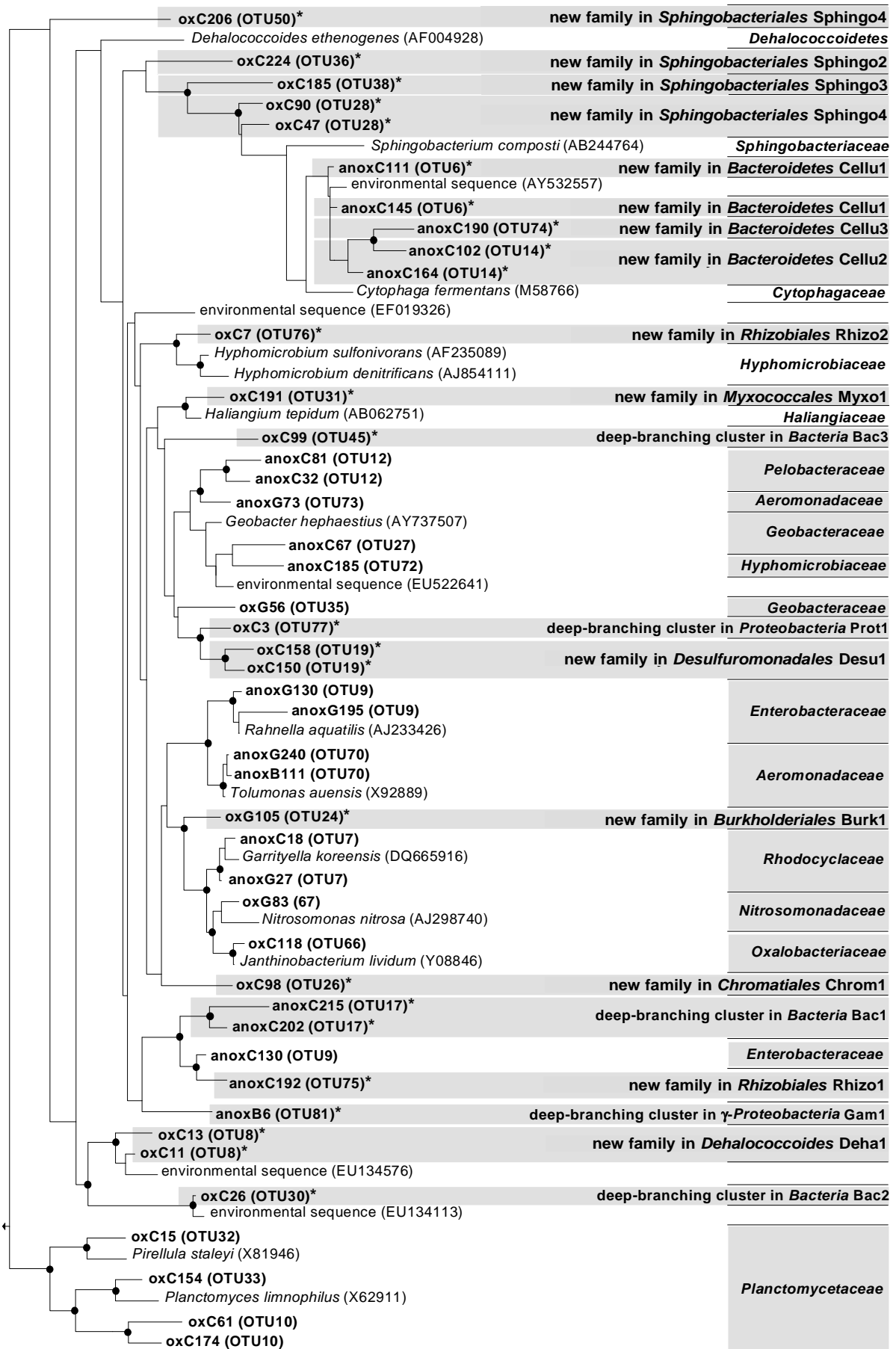


Figure A3. Phylogenetic tree of 16S rRNA cDNA sequences (bold) and reference sequences unrelated to the phyla *Actinobacteria* or *Firmicutes*. Accession numbers are in parentheses, and OTU designations are given in Table 22. Shaded areas highlight labeled taxa. Sequences indicative of new families are marked with an asterisk (*). The tree was calculated with AXML (50% filter; 576 valid positions between positions 130 and 817 of the 16S rRNA gene of *Escherichia coli*). Dots at nodes indicate confirmation of topology by neighbor joining and parsimony using the same dataset. The out group was *Methanosarcina barkeri* (AF028692). Scale bar, 10% evolutionary distance. Abbreviations: ox, oxic microcosm; anox, anoxic microcosm; C, cellulose; B, cellobiose; G, glucose.

Table A1. Numbers of 16S rRNA gene transcripts of detected bacterial taxa in treatments in incubation chambers.^a

Taxon	16S rRNA _{Taxon} [16S rRNA _{Bacteria}] ^{-1 b}				
	0 h	48 h	102 h	168 h	192 h
CMC-supplemented					
<i>Micrococcaceae</i> & <i>Cellulomonadaceae</i>	9.8×10^{-5}	$(5.6 \pm 1.5) \times 10^{-5}$	$(3.6 \pm 1.9) \times 10^{-5}$	4.2×10^{-5}	$(4.8 \pm 1.2) \times 10^{-5}$
<i>Kineosporiaceae</i> & <i>Nocardioideaceae</i>	6.3×10^{-7}	$(3.0 \pm 0.8) \times 10^{-7}$	$(2.1 \pm 1.7) \times 10^{-7}$	3.5×10^{-7}	2.9×10^{-7}
Cluster I <i>Clostridiaceae</i>	4.7×10^{-7}	$(2.3 \pm 2.3) \times 10^{-7}$	$(1.9 \pm 1.5) \times 10^{-7}$	1.7×10^{-7}	$(1.9 \pm 0.8) \times 10^{-7}$
<i>Planctomycetaceae</i>	7.1×10^{-5}	$(3.1 \pm 3.1) \times 10^{-5}$	$(1.6 \pm 1.1) \times 10^{-5}$	1.4×10^{-6}	$(1.8 \pm 0.3) \times 10^{-5}$
'Cellu1-3' ^c	3.7×10^{-7}	2.0×10^{-7}	$(1.0 \pm 0.2) \times 10^{-7}$	4.6×10^{-8}	$(1.3 \pm 1.2) \times 10^{-7}$
'Sphingo1-4' ^c	1.1×10^{-9}	$(4.2 \pm 6.2) \times 10^{-8}$	$(8.4 \pm 0.1) \times 10^{-9}$	2.2×10^{-8}	$(1.0 \pm 1.6) \times 10^{-7}$
'Deha1' ^c	6.8×10^{-4}	$(5.8 \pm 1.6) \times 10^{-4}$	$(3.9 \pm 1.4) \times 10^{-4}$	3.4×10^{-4}	$(4.2 \pm 1.9) \times 10^{-4}$
Cellobiose-supplemented					
<i>Micrococcaceae</i> & <i>Cellulomonadaceae</i>	$(1.1 \pm 0.1) \times 10^{-4}$	$(6.5 \pm 2.1) \times 10^{-3}$	$(6.0 \pm 1.3) \times 10^{-3}$	$(3.6 \pm 0.6) \times 10^{-3}$	$(4.0 \pm 0.7) \times 10^{-3}$
<i>Kineosporiaceae</i> & <i>Nocardioideaceae</i>	$(9.3 \pm 5.8) \times 10^{-7}$	$(2.5 \pm 0.6) \times 10^{-7}$	$(1.3 \pm 0.6) \times 10^{-7}$	2.0×10^{-7}	$(2.0 \pm 0.5) \times 10^{-7}$
Cluster I <i>Clostridiaceae</i>	$(9.4 \pm 3.1) \times 10^{-7}$	$(1.5 \pm 0.7) \times 10^{-6}$	$(1.2 \pm 1.0) \times 10^{-5}$	$(7.5 \pm 6.3) \times 10^{-5}$	$(8.0 \pm 2.5) \times 10^{-5}$
<i>Planctomycetaceae</i>	$(1.2 \pm 0.4) \times 10^{-5}$	$(1.1 \pm 0.5) \times 10^{-6}$	$(1.1 \pm 0.4) \times 10^{-6}$	$(4.5 \pm 2.0) \times 10^{-7}$	$(8.0 \pm 1.3) \times 10^{-7}$
'Cellu1-3' ^c	$(9.0 \pm 2.3) \times 10^{-8}$	$(2.1 \pm 1.0) \times 10^{-7}$	1.6×10^{-6}	1.8×10^{-6}	$(1.6 \pm 0.6) \times 10^{-6}$
'Sphingo1-4' ^c	$(1.1 \pm 0.3) \times 10^{-5}$	$(3.1 \pm 0.9) \times 10^{-6}$	$(2.1 \pm 0.5) \times 10^{-6}$	$(4.5 \pm 0.8) \times 10^{-6}$	$(2.2 \pm 0.3) \times 10^{-6}$
'Deha1' ^c	$(1.3 \pm 0.2) \times 10^{-3}$	$(2.8 \pm 0.4) \times 10^{-4}$	$(2.7 \pm 0.5) \times 10^{-4}$	$(2.0 \pm 1.0) \times 10^{-4}$	$(3.1 \pm 0.3) \times 10^{-4}$

Taxon	16S rRNA _{Taxon} [16S rRNA _{Bacteria}] ⁻¹ ^b				
	0 h	48 h	102 h	168 h	192 h
Unsupplemented control					
<i>Micrococcaceae & Cellulomonadaceae</i>	(2.0 ± 0.4) × 10 ⁻⁴	(1.2 ± 0.2) × 10 ⁻⁴	(1.4 ± 0.4) × 10 ⁻⁴	(1.3 ± 0.2) × 10 ⁻⁴	(8.7 ± 3.5) × 10 ⁻⁵
<i>Kineosporiaceae & Nocardioideaceae</i>	(8.6 ± 4.1) × 10 ⁻⁷	(6.5 ± 1.4) × 10 ⁻⁷	(6.1 ± 0.9) × 10 ⁻⁷	(6.4 ± 1.2) × 10 ⁻⁷	(7.7 ± 5.9) × 10 ⁻⁷
Cluster I <i>Clostridiaceae</i>	(1.0 ± 0.5) × 10 ⁻⁶	(9.9 ± 3.5) × 10 ⁻⁷	(8.7 ± 4.2) × 10 ⁻⁷	(1.4 ± 0.4) × 10 ⁻⁶	(2.4 ± 1.3) × 10 ⁻⁶
<i>Planctomycetaceae</i>	(2.4 ± 0.6) × 10 ⁻⁵	(1.4 ± 0.3) × 10 ⁻⁵	(8.6 ± 2.1) × 10 ⁻⁶	(1.3 ± 0.2) × 10 ⁻⁵	(7.9 ± 3.4) × 10 ⁻⁶
'Cellu1-3' ^c	(4.2 ± 1.6) × 10 ⁻⁸	(5.3 ± 1.1) × 10 ⁻⁸	(4.2 ± 1.2) × 10 ⁻⁸	(4.6 ± 1.0) × 10 ⁻⁸	(5.5 ± 2.8) × 10 ⁻⁸
'Sphingo1-4' ^c	(1.1 ± 0.9) × 10 ⁻⁵	(7.5 ± 4.0) × 10 ⁻⁶	(7.4 ± 2.3) × 10 ⁻⁶	(5.0 ± 0.2) × 10 ⁻⁶	(6.6 ± 3.2) × 10 ⁻⁶
'Deha1' ^c	(9.2 ± 0.9) × 10 ⁻⁴	(7.1 ± 1.3) × 10 ⁻⁴	(6.6 ± 1.4) × 10 ⁻⁴	(8.2 ± 0.9) × 10 ⁻⁴	(5.8 ± 2.3) × 10 ⁻⁴

^a Assignment to a family was based on a minimal 16S rRNA gene similarity of 87% (Yarza et al. 2008).

^b Values are the means of two replicates (without standard deviation) or three replicates (with standard deviation).

^c Name and affiliation as defined elsewhere (3.2.4.2.1).

Bold numbers: The numbers of 16S rRNA gene transcripts that were used as reference (set to 100%) for normalization of relative abundances of sequences of target taxa in Figure 32.

Table A2. Changes in concentration of supplemental cellobiose or glucose and accumulated products between start of incubation and time point at which supplemented sugars were consumed (Concentration data in Figure 25) at 'low' concentration of pesticides^a.

	Difference in concentration [μmol g _{soil} DW ⁻¹]	
	Oxic ^b	Anoxic ^c
Cellobiose		
Bentazon	0.6 ± 0.1	0.6 ± 0.0
MCPA	0.6 ± 0.1	0.6 ± 0.0
Nonylphenol	0.6 ± 0.0	0.6 ± 0.0
Control	0.7 ± 0.1	0.6 ± 0.0
Glucose		
Bentazon	1.2 ± 0.1	0.9 ± 0.2
MCPA	1.3 ± 0.1	0.9 ± 0.0
Nonylphenol	1.1 ± 0.1	0.8 ± 0.0
Control	1.4 ± 0.1	1.2 ± 0.0

	Difference in concentration [$\mu\text{mol g}_{\text{soil DW}}^{-1}$]	
	Oxic ^b	Anoxic ^c
	CO₂	
Bentazon	2.1 ± 1.2	4.1 ± 0.5
MCPA	2.6 ± 0.9	4.1 ± 0.1
Nonylphenol	1.9 ± 0.5	4.6 ± 0.4
Control	2.5 ± 0.7	4.7 ± 0.6
Endogenous CO ₂ production	0.8 ± 0.1	2.6 ± 0.0
	H₂	
Bentazon	-	0.0 ± 0.0
MCPA	-	0.1 ± 0.0
Nonylphenol	-	0.0 ± 0.0
Control	-	1.1 ± 0.0
Endogenous H ₂ production		0.0 ± 0.0

^a Concentration of pesticides was between 0.01 and 0.4 $\mu\text{mol g}_{\text{soil DW}}^{-1}$ (Table 6).

^b Values were calculated between start and 70 days (cellulose supplementation) or between start and 10 hours (cellobiose supplementation).

^c Values were calculated between start and 70 days (cellulose supplementation) or between start and 42 hours (cellobiose supplementation).

Control, pesticides were not added, but substrates were supplemented.

-, Not determined.

Table A3. Changes in concentration of supplemental cellulose, supplemental cellobiose or released glucose and accumulated products between start of incubation and time point at which supplemented sugars were consumed (Concentration data in Figure 26 and Figure 27) at 'high' concentration of pesticides.

	Difference in concentration [$\mu\text{mol g}_{\text{soil DW}}^{-1}$]	
	Oxic ^c	Anoxic ^d
Cellulose-supplemented^a		
	Cellulose	
Bentazon	0.4 ± 0.0	0.7 ± 0.0
MCPA	0.4 ± 0.0	0.6 ± 0.0
Nonylphenol	0.5 ± 0.0	0.6 ± 0.0
Control	0.7 ± 0.1	0.6 ± 0.0
	Glucose	
Bentazon	0.3 ± 0.0	0.2 ± 0.1
MCPA	0.6 ± 0.1	0.3 ± 0.0
Nonylphenol	0.5 ± 0.0	1.2 ± 0.0
Control	1.4 ± 0.1	1.2 ± 0.0
	CO₂	
Bentazon	0.8 ± 0.1	1.7 ± 0.2
MCPA	1.1 ± 0.1	2.3 ± 0.3
Nonylphenol	1.7 ± 0.6	5.8 ± 0.4
Control	2.5 ± 0.7	4.7 ± 0.6
Endogenous CO ₂ production	0.8 ± 0.1	2.6 ± 0.0
	H₂	
Bentazon	-	0.0 ± 0.0
MCPA	-	0.0 ± 0.0
Nonylphenol	-	2.8 ± 0.4
Control	-	1.1 ± 0.0
Endogenous H ₂ production	-	0.0 ± 0.0

	Difference in concentration [$\mu\text{mol g}_{\text{soil DW}}^{-1}$]	
	Oxic ^c	Anoxic ^d
Cellulose-supplemented^b		
	Cellulose	
Bentazon	7.0 ± 1.4	0.6 ± 0.6
MCPA	4.5 ± 3.2	0.7 ± 0.4
Nonylphenol	6.2 ± 1.3	0.3 ± 0.4
Control	11.8 ± 1.7	11.2 ± 1.7
	CO₂	
Bentazon	84.0 ± 6.9	5.5 ± 0.3
MCPA	72.1 ± 4.8	5.2 ± 0.8
Nonylphenol	149.9 ± 14.4	19.9 ± 4.6
Control	105.5 ± 2.6	35.7 ± 3.1
Endogenous CO ₂ production	48.9 ± 5.2	25.1 ± 1.0
	Fe²⁺	
Bentazon	-	7.9 ± 0.9
MCPA	-	8.5 ± 0.5
Nonylphenol	-	74.6 ± 1.9
Control	-	184.9 ± 4.0
Endogenous H ₂ production	-	0.0 ± 0.0

^a Concentration of pesticides was between 2.4 and 8.5 $\mu\text{mol g}_{\text{soil DW}}^{-1}$ (Table 6).

^b Concentration of pesticides was 2.4 $\mu\text{mol g}_{\text{soil DW}}^{-1}$.

^c Values were calculated between start and 70 days (cellulose supplementation) or between start and 10 hours (cellobiose supplementation).

^d Values were calculated between start and 70 days (cellulose supplementation) or between start and 42 hours (cellobiose supplementation).

Control, pesticides were not added, but substrates were supplemented.

-, Not determined.