Functional Redundancy of Anaerobes in Methanogenic Food Webs

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Do not go where the path may lead,

go instead where there is no path and leave a trail.

Ralph Waldo Emerson

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ABBREVIATIONS

atm	standard atmosphere (equals 1.01325 bar)
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bar	unit of pressure
BLAST	basic local alignment search tool
bp	base pair
c	concentration
C	coverage
C/N	carbon to nitrogen ratio
c_S	concentration in slurry
c_{ST}	concentration of standard DNA or of stock solution
c_{TG}	concentration of target gene
CH_3	methyl radical
CH_4	methane
Cl	chlorine
cm	centimeter
CO	carbon monoxide
CO_2	carbon dioxide
CoA	coenzyme A
CODH/ACS	CO dehydrogenase/acetyl-CoA synthase
CoM	coenzyme M
cooS	gene encoding CO dehydrogenase
DNA	deoxyribonucleic acid
DW	dry weight
et al.	*et alii* (translates as ‘and others’)
e.g.	*exempli gratia* (translates as ‘for example’)

FHL	formate-hydrogenlyase
fhs	gene encoding formyltetrahydrofolate synthetase
FW	fresh weight
g	gram
g	relative centrifugal force (equals 9.81 m s^{-2})
$G_f°$	standard Gibbs energy of formation
H_2	molecular hydrogen
H_2ase	hydrogenase
HCl	hydrochloric acid
H_4MPT	tetrahydromethanopterin
H_2O	water
hPa	hectopascal
H_4SPT	tetrahydrosarcinapterin
i.e.	*id est* (translates as ‘in other words)
K	kelvin
K'	equilibrium constant
kg	kilogram
kJ	kilojoule
km^2	square kilometer
L	liter
m	meter
m	amount of compound
M	molar
mbar	millibar
mcrA	gene encoding the alpha-subunit of methyl-CoM reductase
mg	milligram
mL	milliliter
mm	millimeter

mM	millimolar
mmol	millimole
mol	mole
MPN	most probable number
mV	millivolt
MW	molecular weight
n	single sequence
n_C	amount of chemically dissolved CO_2
n_G	amount of gas in a gas phase
n_L	amount of physically dissolved gas in a liquid phase
n_T	total amount of gas
n_{TG}	length of the target gene
N	normal
N	total number of sequences or rotational speed
N_A	Avogadro constant (equals 6.23 x 10^{23} molecules mol^{-1})
N_2	dinitrogen
ng	nanogram
nm	nanometer
N_2O	dinitrogen monoxide
NO_x	molecule with one nitrogen atom and an unspecified number of oxygen atoms
NPOC	non-purgeable organic carbon
O_2	molecular oxygen
p	partial pressure of a gas
p_A	actual atmospheric pressure
p_O	overpressure in an incubation flask
pKa	acid dissociation constant
PCR	polymerase chain reaction

pH	the negative decimal logarithm of hydrogen ion activity in a solution
pg	picogram (equals 10^{-12} g)
Pg	petagram (equals 10^{15} g)
ppm	parts per million
qPCR	quantitative polymerase chain reaction
r	rotational radius
R	gas constant (equals 8.31 J mL^{-1} K^{-1} or 83.145 mbar mL K^{-1} $mmol^{-1}$)
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolution per minute
s	standard deviation
SIP	stable isotope probing
soil	Introduction: soil that may contain roots (no specification in literature); all other chapters: root-free soil
‘soil’	soil that contains roots
sp.	species
T	actual temperature
Tg	terragram (equals 10^{12} g)
U	units
UV	ultraviolet
V_L	volume of a liquid phase
V_S	volume of stock solution
V_T	total volume of a slurry
w/v	weight per volume
X	rate of the gas
yr	year
at %	atomic percent

$\bar{x}$	mean value of all samples
%	percent
°C	degree centigrade
µl	microliter
µm	micrometer
µM	micromolar
µmol	micromole
λ	solubility coefficient
ΔG	change of the Gibbs free energy
$\Delta G^{\circ\prime}$	change of Gibbs free energy under standard conditions
^{12}C	most common carbon isotope on earth
^{13}C	heavy isotope of the element carbon
^{14}C	radioactive isotope of the element carbon

1 Summary

Methane (CH_4) is an important greenhouse gas and is predominantly produced by methanogens. The production of CH_4 is driven by a stepwise degradation of organic matter into intermediates by a complex microbial food web in which methanogenesis is the terminal process. This food web trophically links fermentation, syntrophic fermentation, acetogenesis, and methanogenesis. However, methanogenic habitats can differ in pH, temperature, and availability of nutrients and carbon sources, and thus, may harbor dissimilar microbial communities that are adapted to those varying conditions and collectively catalyze the degradation of complex organic matter. In other words, methanogenic food webs of different CH_4-emitting habitats may be driven by functionally redundant anaerobes. Despite these theoretical considerations, methanogenic food webs are for the most part conceptualized rather than resolved. The objectives of this dissertation were to (a) resolve the complex methanogenic food webs of contrasting CH_4-emitting habitats and (b) determine if those methanogenic food webs are driven by functionally redundant anaerobes.

The objectives were addressed with cultivation-dependent, analytical, and molecular approaches, including isolation, supplementation of anoxic slurries, determination of dissimilation products, quantification of cultivable microorganisms, stable isotope probing, quantification of gene copy numbers, analysis of 16S rRNA and 16S rRNA genes, and analysis of structural genes. Soil from four contrasting mires, root-free soil and soil-free roots of mire plants, and gut contents of the earthworm *Eudrilus eugeniae* were analyzed.

Contrasting mire soils showed similar glucose-, acetate-, and H_2-CO_2-dependent product profiles, cell numbers of cultivable microorganisms, and gene copy numbers, but major differences were observed in bacterial and methanogenic communities. Only 15 % of species-level *mcrA* and family-level 16S rRNA gene phylotypes (*mcrA* encodes the alpha-subunit of methyl-CoM reductase) were common to all mire soils, indicating that methanogenic food webs are for the most part driven by dissimilar microorganisms. For example, *Clostridiaceae* were

common to all mire soils whereas *Bacillaceae* or *Peptococcaceae* were restricted to only one of the mire soils. About 40 % of the detected family-level phylotypes of each mire soil have no cultured isolate, illustrating that a diverse array of mire-derived microorganisms await characterization. The majority of taxa detected in acidic mire soils were also detected in more neutral mire soils whereas many taxa detected in more neutral mire soils were not detected in acidic mire soils, suggesting that pH restricted the diversity of microorganisms in acidic mire soils.

Formate can be a substrate for methanogens and is released from mire-derived fermenters and from plant roots as a root exudate, suggesting that the root zone might be a hot spot for methanogenesis. Surprisingly, soil-free roots of *Molinia caerulea* and *Carex* sp. from one of the aforementioned mires initially produced H_2-CO_2 in response to supplemental formate. Two isolates related to *Citrobacter* and *Hafnia* were obtained from those roots and were capable of fermentation. Both isolates catalyze the formation of H_2 from formate via the formate-hydrogenlyase complex harboring a group 4 [NiFe]hydrogenase. The production of CH_4 and acetate by methanogens and acetogens, respectively, occurred subsequently either indirectly from formate-derived H_2 or directly from supplemental formate. These observations illustrate a potential trophic interaction between formate-hydrogenlyase-containing fermenters, acetogens, and methanogens.

Gut contents of the CH_4-emitting earthworm *E. eugeniae* fermented glucose, produced acetate from H_2-CO_2 via acetogenesis, and produced CH_4. A methanogenic and an acetogenic enrichment were obtained from gut contents. The methanogenic enrichment utilized formate and H_2-CO_2 and contained species of *Methanobacterium*. The acetogenic enrichment formed acetate from formate and H_2-CO_2 in a stoichiometric ratio indicative of acetogenesis and contained an acetogen related to *Terrisporobacter*. Most detected fermenters, acetogens, and methanogens differed from taxa detected in mire soils and on mire-derived plant roots.

Fermenters and acetogens were isolated or enriched from aerated forest soil, roots of mire plants, and gut contents of the earthworm *E. eugeniae*. The fermenters produced

intermediates that were consumed by the acetogens. For example, the *Clostridium*-related acetogen SB1 was enriched in a culture together with the *Clostridium*-related fermenter SB3 and the *Carnobacterium*-related fermenter SB4 from roots of mire plants. Both fermenters were obtained in pure culture and produced H_2, ethanol, formate and lactate from glucose under anoxic conditions. In contrast, in co-culture with the *Clostridium*-related acetogen SB1, only acetate was produced from glucose, indicating that the acetogen utilized the fermentation products of the two fermenters. These findings illustrate the potential trophic interactions and functional redundancy of fermenters and acetogens in contrasting habitats.

The collective results indicated that microbial processes driving the methanogenic food webs in mire soils, the rhizosphere of mire plants, and gut contents of *E. eugeniae* are qualitatively more similar than dissimilar but are facilitated by dissimilar microbial communities. The functional redundancy of the microbial communities is in particular reflected by the large number of detected taxa not identical in each of the anoxic habitats but nonetheless catalyzing similar processes.

2 Zusammenfassung

Methan (CH_4) ist ein wichtiges Treibhausgas, welches vor allem von Methanogenen produziert wird. Die Produktion von CH_4 ist von dem schrittweisen Abbau organischen Materiales in Intermediate angetrieben. Der Abbau wird von einem komplexen mikrobiellen Nahrungsnetz mit Methanogenese als abschließendem Prozess katalysiert. Dieses Nahrungsnetz verbindet Gärung, syntrophe Gärung, Acetogenese und Methanogenese. Methanogene Habitate variieren jedoch in pH, Temperatur, Verfügbarkeit von Nährstoffen und Kohlenstoffquellen, und könnten daher unterschiedliche mikrobielle Gesellschaften aufweisen, welche an die verschiedenen Bedingungen angepasst sind und gemeinsam den Abbau von komplexem, organischem Materials katalysieren. Das heißt, methanogene Nahrungsnetze in unterschiedlichen CH_4-emittierenden Habitaten könnten durch funktionell redundante Anaerobe angetrieben werden. Unabhängig von theoretischen Betrachtungen sind methanogene Nahrungsnetze größtenteils konzipiert und wenig aufgeklärt. Die Zielsetzung dieser Dissertation war es (a) komplexe methanogene Nahrungsnetze von unterschiedlichen CH_4-emittierenden Habitaten aufzuklären und (b) zu bestimmen, ob diese methanogenen Nahrungsnetze von funktionell redundanten Anaeroben angetrieben sind.

Die Fragestellung wurde mit kultivierungsabhängigen, analytischen und molekularen Methoden bearbeitet, welche Isolierung, Supplementierung von anoxischen Aufschlämmungen, Bestimmung von Dissimilationsprodukten, Quantifizierung von kultivierbaren Mikroorganismen, Beprobung von stabilen Isotopen, Quantifizierung von Kopienzahlen von Genen, Analyse von 16S rRNA und 16S rRNA Genen, und Analyse von strukturellen Genen umfasste. Der Boden von vier unterschiedlichen Mooren, wurzelfreier Boden und bodenfreie Wurzeln von Moorpflanzen, und der Darminhalt des Regenwurmes *Eudrilus eugeniae* wurden analysiert.

Die unterschiedlichen Moorböden wiesen ähnliche Glukose-, Acetat- und H_2-CO_2-abhängige Produktprofile, ähnliche Zellzahlen kultivierbarer Mikroorganismen und ähnliche

Kopienzahlen von Genen auf. Die bakteriellen und methanogenen Gesellschaften zeigten hingegen große Unterschiede. Lediglich 15 % der *mcrA* Phylotypen auf Speziesebene (*mcrA* kodiert die Alphauntereinheit der Methyl-CoM Reduktase) und 16S rRNA Gen Phylotypen auf Familienebene konnten in allen Moorböden nachgewiesen werden, was auf methanogene Nahrungsnetze hindeutet, die größtenteils durch unterschiedliche Mikroorganismen angetrieben werden. *Clostridiaceae* beispielsweise wrden in allen Moorböden detektiert wohingegen *Bacillaceae* oder *Peptococcaceae* begrenzt auf nur einen der Moorböden waren. Von jedem Moorboden waren etwa 40 % der detektierten Phylotypen auf Familienebene mit keinem kultivierten Isolat assoziiert. Dies veranschaulicht das breite Spektrum an Mikroorganismen in Moorböden, welches es noch zu charakterisieren gilt. Der Großteil der Taxa, der in sauren Moorböden detektiert wurde, wurde auch in eher neutraleren Moorböden detektiert. Viele Taxa, die in eher neutraleren Moorböden detektiert wurden, wurden nicht in sauren Moorböden detektiert. Diese Beobachtungen sind ein Hinweis darauf, dass die Diversität an Mikroorganismen in sauren Moorböden durch den pH eingeschränkt wurde.

Formiat kann als Substrat von Methanogenen verwendet werden und wird von Gärern, die in Moorböden zu finden sind, und von Pflanzenwurzeln in Form von Wurzelexsudaten freigesetzt, was auf den Wurzelbereich als Hotspot für Methanogenese hinweisen könnte. Überraschenderweise produzierten bodenfreie Wurzeln von *Molinia caerulea* und *Carex* sp. aus einem der zuvor genannten Moore zunächst H_2-CO_2 als Reaktion auf die Zugabe von Formiat. Zwei Isolate wurden von genannten Wurzeln gewonnen, welche mit *Citrobacter* und *Hafnia* verwandt sind und gären können. Beide Isolate katalysieren die Bildung von H_2 aus Formiat mittels des Formiat-Hydrogenlyase-Komplexes, welcher eine Gruppe 4 [NiFe]Hydrogenase enthält. Die Produktion von CH_4 und Acetat durch Methanogene und Acetogene trat nach der Produktion von H_2 auf, entweder indirekt von aus Formiat gebildetem H_2 oder direkt von zugegebenem Formiat. Diese Beobachtungen veranschaulichen potentielle trophische Interaktionen zwischen Acetogenen, Methanogenen und Gärern, die einen Formiat-Hydrogenlyase-Komplex besitzen.

Der Darminhalt des CH_4-emittierenden Regenwurmes *E. eugeniae* fermentierte Glukose, produzierte H_2-CO_2 aus Acetat durch Acetogenese und produzierte CH_4. Eine methanogene und eine acetogene Anreicherungskultur wurden aus dem Darminhalt gewonnen. Die methanogene Anreicherungskultur nutzte Formiat und H_2-CO_2 und beinhaltete Spezies der Gattung *Methanobacterium*. Die acetogene Anreicherungskultur bildete Acetat aus Formiat und H_2-CO_2 in einem stöchiometrischen Verhältnis, welches auf Acetogenese hinwies, und beinhaltete einen zu *Terrisporobacter* verwandten Acetogenen. Die meisten detektierten Gärer, Acetogenen und Methanogenen unterschieden sich von Taxa, die in Moorböden oder an Wurzeln von Moorpflanzen gefunden wurden.

Gärer und Acetogene wurden aus belüftetem Waldboden, Wurzeln von Moorpflanzen und dem Darminhalt des Regenwurmes *E. eugeniae* isoliert oder angereichert. Die Gärer produzierten Intermediate, welche von den Acetogenen konsumiert wurden. Der Acetogene SB1 (verwandt zu *Clostridium*) beispielsweise wurde in einer Kultur zusammen mit den Gärern SB3 (verwandt zu *Clostridium*) und SB4 (verwandt zu *Carnobacterium*) aus Wurzeln von Moorpflanzen angereichert. Beide Gärer wurden in Reinkultur gewonnen und produzierten unter anoxischen Bedingungen H_2, Ethanol, Formiat und Laktat aus Glukose. Im Kontrast dazu, produzierte die Kokultur mit dem Acetogenen SB1 lediglich Acetat von Glukose. Dies weist darauf hin, dass der Acetogene SB1 Gärungsprodukte der beiden Gärer nutzen kann. Diese Beobachtungen veranschaulichen die möglichen trophischen Interaktionen von Gärern und Acetogenen in unterschiedlichen Habitaten.

Die Gesamtheit der Ergebnisse zeigt, dass sich die mikrobiellen Prozesse, welche die methanogenen Nahrungsnetze in Moorböden, der Rhizosphäre von Moorpflanzen und dem Darminhalt von *E. eugeniae* antreiben, qualitativ stärker ähneln als unterscheiden, jedoch von unterschiedlichen mikrobiellen Gesellschaften ermöglicht werden. Die funktionelle Redundanz mikrobieller Gesellschaften ist besonders durch die große Anzahl detektierter Taxa reflektiert, welche in den jeweiligen anoxischen Habitaten nicht identisch sind, aber dennoch ähnliche Prozesse katalysieren.

3 Introduction

Methane (CH_4) is an important greenhouse gas and is predominantly produced by methanogens in various anoxic habitats when carbon dioxide (CO_2) is the main terminal electron acceptor (Bouwman 1990, Thauer 1998, Dianou *et al.* 2001, Mizukami *et al.* 2006). The production of CH_4 is driven by a stepwise degradation of organic biopolymers into intermediates by a complex microbial food web in which methanogenesis is the terminal process (Zehnder 1978, McInerney and Bryant 1981, Drake *et al.* 2009). This food web trophically links fermentation, syntrophic fermentation, acetogenesis, and methanogenesis (Zehnder 1978, McInerney and Bryant 1981, Drake *et al.* 2009), suggesting that similar processes fuel methanogenesis in different anoxic habitats. However, methanogenic habitats can differ in pH, temperature, and availability of nutrients and carbon (Westermann 1993, Ding *et al.* 2002, Wüst *et al.* 2009b), and thus may harbor contrasting microbial communities that are adapted to those varying conditions and collectively catalyze the degradation of complex organic biopolymers. In other words, methanogenic food webs of different CH_4-emitting habitats may be driven by functionally redundant anaerobes (i.e., by microorganisms that are identical in function but phylogenetically distinct). Despite these theoretical considerations, methanogenic food webs are for most part conceptualized in textbooks rather than resolved in the primary literature (Drake *et al.* 2009). The focus of this dissertation was to (a) resolve the complex methanogenic food webs of contrasting CH_4-emitting habitats and (b) determine if those methanogenic food webs are driven by functionally redundant anaerobes.

3.1 Greenhouse gases contribute to global warming

Thirty percent of the energy of sunlight is reflected back into the solar system whereas the remaining 70 % are absorbed by molecules in the atmosphere and on the Earth's surface (Rogers and Whitman 1991, Stocker *et al.* 2013). Molecules in the atmosphere such as water, CO_2, and CH_4 are very effective in absorbing the energy of sunlight, and reflecting a part of it back into the solar system or towards the Earth's surface (Rogers and Whitman 1991). CH_4

is the most abundant hydrocarbon in the atmosphere and can be chemically oxidized to water vapor and CO_2 (Bouwman 1990, Rogers and Whitman 1991). The oxidation of CH_4 results in the production of ozone under high NO_x concentrations and for example occurs in polluted air in the troposphere (Rogers and Whitman 1991). Consequently, increasing concentrations of CH_4 positively influence the concentration of toxic tropospheric ozone, and thus contributes to air pollution (West *et al.* 2006). CH_4 also reacts with chlorine (Cl) in the atmosphere and forms hydrogen chloride (HCl) and CH_3 (Bouwman 1990, Rogers and Whitman 1991). CH_3 is further oxidized whereas HCl is rained out of the stratosphere as acidic rain (Rogers and Whitman 1991). The absorption of sun energy is essential to maintain an average global temperature that makes life possible on earth (i.e., approximately 15 °C) (Rogers and Whitman 1991).

CO_2 and CH_4 are the two most important greenhouse gases with the greatest impact on global warming (Andreae and Crutzen 1985, Bouwman 1990). For example, CO_2 and CH_4 account for 82 % and 10 % of total emitted greenhouse gases in the United States, respectively (Leaf *et al.* 2003). Two hundred years ago, the concentration of atmospheric CO_2 was about 280 ppm and has been increasing since (Barnola *et al.* 1987, Stocker *et al.* 2013). In 2011, CO_2 concentration approximated 391 ppm (Stocker *et al.* 2013). The increase of CO_2 in the atmosphere is a result of human activity and mostly due to fossil fuel burning (Rogers and Whitman 1991, Stocker *et al.* 2013). In 1750, the concentration of CH_4 was 0.7 ppm and had increased up to 1.8 ppm in 2011 (Stocker *et al.* 2013). Between 1980 and 2006 the annual emission of CH_4 remained fairly stable with approximately 550 Tg CH_4 per year but started to increase again since 2007 (Stocker *et al.* 2013). Sources of CH_4 are natural wetlands, agriculture and waste, ruminants, fossil fuel related emissions, biomass and biofuel burning (Liu and Whitman 2008, Stocker *et al.* 2013, Moore *et al.* 2014). Anthropogenic emissions account for approximately 50 % of total emissions (Stocker *et al.* 2013). Microbially mediated CH_4 production occurs in various anoxic habitats under the absence of O_2 and other electron acceptors except CO_2 (Balch *et al.* 1979, Rogers and Whitman 1991, Ding *et al.* 2002). Natural wetlands are the single most important source of CH_4 and account for approximately 187-224 Tg CH_4 yr^{-1} which is 34-41 % of total CH_4 emission (Liu and Whitman 2008, Stocker *et al.*

2013). It is predicted that a rising concentration of greenhouse gases in the atmosphere will increase global warming and thus being the trigger to increasing global temperature, rising sea level, diminishing snow and ice covers, increasing extreme weather, and increasing acidification of the oceans (Rogers and Whitman 1991, Stocker *et al.* 2013).

The average global temperature increased 0.6 °C per 10 years over the last 30 years in high-latitude regions of the Earth, regions that include most of the permafrost-influenced soils (Schuur *et al.* 2015). Normally frozen soil thaws and stored recalcitrant carbon is decomposed by trophically linked microbial food webs and released into the atmosphere in form of CO_2 and CH_4 (Schuur *et al.* 2015). The total global organic carbon pool in soil is estimated to be 2,344 Pg carbon in the top three meters (Jobbágy and Jackson 2000). In comparison, the organic carbon pool in soil of the northern permafrost soil is estimated to be 1,035 Pg carbon in the top three meters (Schuur *et al.* 2015), illustrating that approximately 45 % of the total global organic carbon pool is stored in northern permafrost soils and theoretically can be released as CO_2 and CH_4 when frozen soils thaw and contribute to global warming.

3.2 Natural wetlands: A source of CH_4

Wetlands are distinguished from other terrestrial ecosystems by having (a) a water table near the land surface, (b) unique soil conditions that are strongly influenced by the limited availability of molecular oxygen (O_2), and (c) a specialized biota that is characterized by plants and other organisms that are adapted to wet and reduced soils (Charman 2002, Rydin and Jeglum 2006). Peatland, mires, fens, and bogs are specific types of wetlands (Crum 1992, Westermann 1993, Charman 2002). Mires include fens and bogs, and peat-forming mires are often called peatlands (Charman 2002). Peat consists predominantly of remains from plants (Gorham 1991). Fens and bogs are characterized by pore water with a low pH, low concentrations of ions, and low availability of O_2 (Crum 1992, Westermann 1993). Bogs have a lower pH and lower concentrations of ions than fens (Crum 1992, Westermann 1993). Water and nutrients in fens derive from precipitation and other sources such as ground water, and in

bogs solely from precipitation (Charman 2002). The vegetation of fens and bogs differs (Gorham 1991, Crum 1992). Species of *Carex* are more often found in fens and species of *Sphagnum* are more often found in bogs (Gorham 1991, Crum 1992). It has been observed that the pH is influenced by the growth rate of *Sphagnum* moss and becomes more acidic with higher growth rates (Charman 2002). On a dry weight basis, *Sphagnum* moss consists to 10-30 % of uronic acids (i.e., sugar acids with a carboxyl group) (Charman 2002). The carboxyl group removes cations from the environment of the *Sphagnum* moss and thus contributes to low ion concentrations in bogs (Charman 2002).

Peatlands are especially interesting in regard to greenhouse gases. On one hand, peatlands emit considerable amounts of CH_4 and on the other hand store CO_2 from the atmosphere in the form of peat (Gorham 1991). The area of peatland is estimated to be about 4×10^6 km^2 worldwide, which is about 2.3 % of the earth's terrestrial surface and most parts of it are distributed on the northern hemisphere (Gorham 1991, Immirzi *et al.* 1992, Charman 2002). In Germany alone, an area of about 15×10^3 km^2 is covered by peat, which is about 4.3 % of the total area of Germany (Montanarella *et al.* 2006). Despite this relatively small area, peatlands of the northern hemisphere store about one third (i.e., 455 Pg carbon) of the total global pool of soil carbon and contribute considerably to the emission of CH_4 (Gorham 1991, Stocker *et al.* 2013).

CH_4 is produced from methanogens that have been studied in diverse wetlands (Großkopf *et al.* 1998, Bräuer *et al.* 2004, Chin *et al.* 2004, Cadillo-Quiroz *et al.* 2006, Wüst *et al.* 2009a, Lin *et al.* 2014b). Methanogens of such habitats consume predominately H_2-CO_2 and acetate that can be produced by fermentation (Chin *et al.* 2004, Bräuer *et al.* 2004, Drake *et al.* 2009). The trophic interactions between fermenters and methanogens in wetland soils are less studied in comparison to CH_4 emission and methanogenesis, and are for most parts conceptualized.

3.3 Plants influence the emission of CH_4 in wetland soils

Vegetation influences the emission of CH_4 in water-saturated soils (Koelbener *et al.* 2010, Williams and Yavitt 2010), and up to 90 % of the emitted CH_4 might be facilitated by plants (Watanabe *et al.* 1999, Colmer 2003) (Figure 1). For example, rice paddy soils have higher CH_4 emission rates than paddy soils without plants (Dannenberg and Conrad 1999). The capacity of plants to transport CH_4 from the roots to the atmosphere varies depending on season and plant species (Ding *et al.* 2002, Ding and Cai 2003, Laanbroek 2010). For instance, species of *Carex* increase the emission of CH_4 from wetland soils more than other typical wetland plants (Ding *et al.* 2002, Ström *et al.* 2005, Kao-Kniffin *et al.* 2010, Koelbener *et al.* 2010,). The emission of CH_4 increases with increasing water-table, above ground plant biomass, stem density, density of plant roots, plant litter, and availability of labile organic carbon (Jobbágy and Jackson 2000, Joabsson and Christensen 2001, Ding *et al.* 2002). While stem density increases with increasing above ground plant biomass, and labile organic carbon increases with depth, density of plant roots, water-table, and plant litter (Jobbágy and Jackson 2000, Joabsson and Christensen 2001, Ding *et al.* 2002). With freshwater marshes, it has been observed that the emission of CH_4 is driven by the availability of labile organic carbon, whereas the availability of labile organic carbon is rather influenced by high amounts of plant litter and a high water table than by photosynthetically active *Carex* and *Deyeuxia* plants and above ground biomass (Ding *et al.* 2002). With paddy soil, it has been observed that up to 70 % of the emitted CH_4 derived from exudates of the rice plant (Watanabe *et al.* 1999), indicating that plant species influences the carbon source for methanogenesis.

Plants influence the emission of CH_4 by (a) releasing organic carbon into the soil that can fuel methanogenesis (Jones 1998, Ström *et al.* 2003), (b) transporting CH_4 from the soil into the atmosphere via the aerenchyma in roots, stems, and leaves (Verville *et al.* 1998), and (c) leaking O_2 into the rhizosphere, and thus, inhibiting methanogenesis and stimulating methanotrophy (Van der Nat and Middelburg 1998, Armstrong *et al.* 2000, Laanbroek 2010, Lamers *et al.* 2013). The aerenchyma is a special tissue in roots, stems, and leaves of plants in water-saturated soils allowing the diffusion of O_2 from leaves into roots and the diffusion

from CO_2 and CH_4 from roots into leaves and from there into the atmosphere (Armstrong 1979, Colmer 2003, Ding and Cai 2003, Laanbroek 2010). Up to 40 % of O_2 which diffuses from leaves to roots for root respiration is lost to soil and may form an oxic zone around the root (Colmer 2003). Obligate aerobes such as the methylotrophs *Methylomonas*, *Methylobacter*, *Methylococcus* (Horz *et al.* 2001) and obligate anaerobes such the methanogens *Methanosarcinaceae*, *Methanobacteriaceae*, and *Methanocellaceae* can be detected on roots of rice plants (Chin *et al.* 2004), indicating that the rhizosphere of wetland plants offer habitats for microorganisms that require different O_2 availabilities.

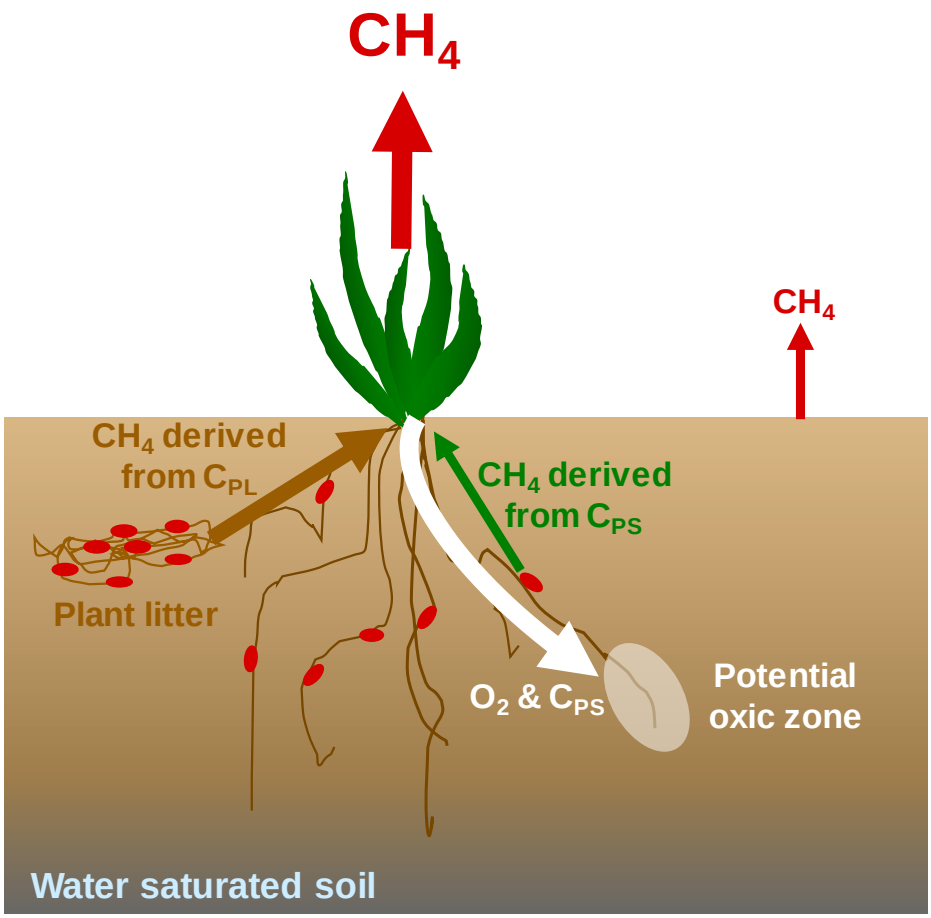

Figure 1: Theoretical model of the emission of CH_4 in water-saturated soils.

Red rods indicate methanogens. A thicker arrow indicates a potentially more important flux than a thinner arrow. Legend: C_{PL}, plant litter-derived carbon; C_{PS}, photosynthesis-derived carbon. Model was created based on observations made by Watanabe *et al.* (1999), Colmer (2003), Koelbener *et al.* (2010), and Williams and Yavitt (2010).

The term rhizosphere derives from the Greek and descripts the 'influence of a root on its surrounding' and is generally the zone of soil that is subjected to the influence of the living

plant root (Brimecombe *et al.* 2001, Pinton *et al.* 2001). Compounds that are released by the root are called exudates (Walker *et al.* 2003). Soil within two millimeters of the root can be affected by plant exudates and plant-derived O_2 (Armstrong *et al.* 2000, Pinton *et al.* 2001). Root exudates constitute a smorgasbord of substrates for microorganisms and include polysaccharides, sugars, amino acids, organic acids, fatty acids, sterols, phenols, growth factors, enzymes, flavones, nucleotides, and mucilage (Brimecombe *et al.* 2001, Uren 2001, Walker *et al.* 2003). Such compounds are released actively and controlled by the roots (Jones 1998, Pinton *et al.* 2001, Bais *et al.* 2006). Passive leakage including the release of sugars approximates five percent of plant-fixed carbon (Uren 2001, Lambers *et al.* 2009). Thirty to sixty percent of the carbon fixed by photosynthesis can be translocated to the roots and up to 70 % of carbon in roots can be released into rhizospheric soil (Coleman 1976, Neumann and Römheld 2001, Pinton *et al.* 2001, Uren 2001). The amount of compounds released depends on plant age, plant species, light intensity, soil type, nutritional status of the plant, stress factors, mechanical impedance, temperature, and microbial activity in the rhizosphere (Brimecombe *et al.* 2001, Pinton *et al.* 2001, Koelbener *et al.* 2010). Organic carbon can be released in concentrations of 10-250 mg g[root]$^{-1}$ and enhances the growth of microorganisms in rhizospheric soil (Brimecombe *et al.* 2001, Lambers *et al.* 2009). Generally, microbial activity and biomass is higher in the rhizosphere (e.g., 10^9 to 10^{12} cells g[rhizosphere soil]$^{-1}$) than in bulk soil (Brimecombe *et al.* 2001, Pinton *et al.* 2001).

Microorganisms mediate the turnover of carbon, nitrogen and other nutrients as well as the mineralization of organic compounds in soil, and thus, enhance the growth of plants (Brimecombe *et al.* 2001). Plants can be highly selective for their microbial community in the rhizosphere and thus improve their health (Brimecombe *et al.* 2001). Plants influence their environment including microorganisms by specific compounds that are actively released from roots (Jones 1998, Brimecombe *et al.* 2001, Bais *et al.* 2006). Such compounds may (a) chelate metals for detoxification (e.g., aluminum) or mobilize nutrients (e.g., phosphorus, iron [Lambers *et al.* 2009]), (b) attract beneficial microorganisms (Döbereiner and Pedrosa 1987, Bais *et al.* 2006), (c) increase the capacity of soil to hold water (Walker *et al.* 2003), (d) enable

communication with other plants (e.g., release of phytotoxins that inhibit growth of neighboring plants [Bais *et al.* 2006]), and (e) enable communication to microorganisms (e.g., plant exudates mimic quorum sensing of bacteria [Bais *et al.* 2006]). Beneficial microbes can be N_2-fixing *Proteobacteria* (Döbereiner and Pedrosa 1987, Bais *et al.* 2006), bacteria that produce antibiotics against potential pathogens (Bais *et al.* 2006, Lambers *et al.* 2009), or sulfide-oxidizing bacteria (Friedrich *et al.* 2001, Lamers *et al.* 2013).

3.4 Earthworms: A source of CH_4?

Earthworms belong to the class *Oligochaeta* and inhabit terrestrial habitats (Edwards 2004). Species that measure a few millimeters and species that measure more than one meter are known (Lee 1985, Edwards and Bohlen 1996, Makeschin 1997). Earthworms ingest 1-30 times the fresh weight of their own body per day and have an average gut passage time of 2-24 hours (Brown *et al.* 2000). Soil and organic matter that passes through the earthworm is reconstructed in its physical, chemical, and biological properties (Brown *et al.* 2000). Consequently, earthworms interact and affect soil organisms, for example, by (a) exploiting soil organic matter that can be utilized by other organisms or (b) forming burrows that aerate the soil (Lavelle 1986, Brown 1995, Brown *et al.* 2000).

Earthworms have specific feeding preferences. Epigeic earthworms such as *Eudrilus eugeniae* feed on and dwell in litter but do not form burrows (Bouché 1977, Lavelle 1981, Brown 1995, Brown *et al.* 2000). *Eudrilus eugeniae* is native to certain African soils and is used commercially in vermicomposting systems in other parts of the world (e.g., processing of cow manure and sugar cane in Brazil [Martinez 1998, Domínguez 2004; Oboh *et al*. 2007]). Anecic earthworms such as *Lumbricus terrestris* feed on litter and soil, dwell in soil, and form vertical burrows (Bouché 1977, Lavelle 1981, Brown 1995). Endogeic earthworms such as *Pontoscolex corethrurus* feed on and dwell in soil, and form horizontal burrows (Bouché 1977, Lavelle 1981, Brown 1995). They also graze on rhizospheric soil (Doube and Brown 1998) that is richer in plant-derived organic carbon and has a higher abundance of microorganisms than bulk soil (Brimecombe *et al.* 2001, Neumann and Römheld 2001, Pinton *et al.* 2001, Uren

2001). Earthworm activity has been shown to decrease diseases of plant roots (Doube and Brown 1998). Plants benefit from burrows and cast in burrows (Brown *et al.* 2000). Burrows deliver O_2 to roots and provide little resistance to growing roots in comparison to compact soil (Brown *et al.* 2000). Cast represents an easily available source of nutrients for plants (Brown *et al.* 2000). Earthworms distribute beneficial microorganisms such as species of N_2-fixing *Rhizobium* (Stephens *et al.* 1994).

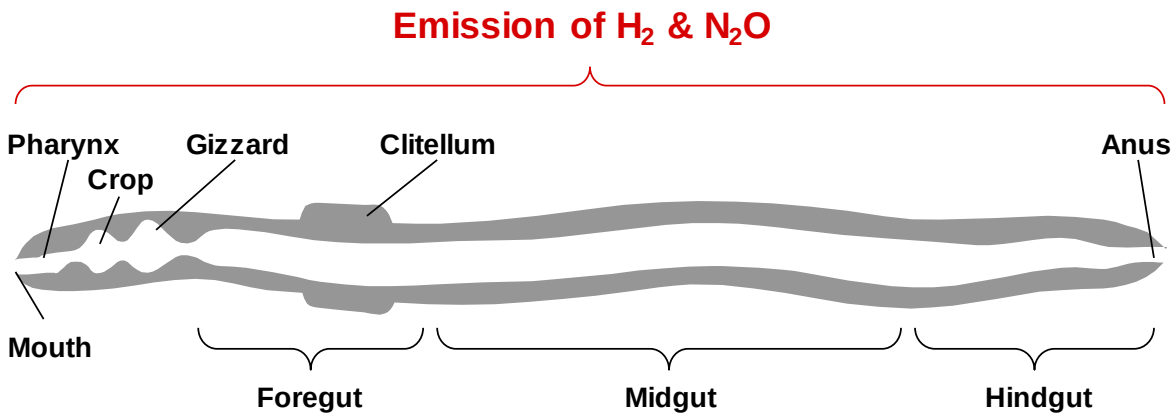

Figure 2: Anatomy of the digestive system of an earthworm.

The figure was modified from Horn *et al.* (2003b) and Wüst *et al.* (2009b) and is based on knowledge gained from *L. terrestris*.

Earthworms ingest soil, organic matter, fungi, protozoa, and other organisms (Brown *et al.* 2000). Those substrates pass pharynx, crop, and gizzard (Brown *et al.* 2000). Ingested material such as soil, fungal hyphae, large bacteria, and algae are ground and mixed in crop and gizzard (Brown *et al.* 2000). In the foregut, a high amount of water and a carbon-rich secrete called mucus is provided to the ingested organic matter, and the pH is neutralized (Barois and Lavelle *et al.* 1983, Lavelle 1986, Martin *et al.* 1987, Trigo and Lavelle 1993). Most digestion occurs in midgut and hindgut where bacteria become activated by supplements of the foregut (e.g., water, carbon) and metabolize the ingested organic matter (Parle 1963, Brown *et al.* 2000). Water, nutrients, and organic carbon are assimilated by the earthworm in the hindgut (Brown *et al.* 2000).

Different earthworm species produce mucus with a similar carbon and nitrogen content, indicating that mucus of different earthworm species is constructed similarly (Brown *et al.*

2000). Mucus consists of amino acids, organic acids, amino sugars, sugars, and glycoproteins (Rahemtulla and Løvtrup 1975, Martin *et al.* 1987, Drake and Horn 2007, Wüst *et al.* 2009b). For example, glucose, maltose, formate, and acetate were detected in the gut of earthworms (Drake and Horn 2007, Wüst *et al.* 2009b). The amount of mucus decreases during gut passage from the anterior to the posterior end of the earthworm (Trigo *et al.* 1999). Epigeic and anecic earthworms have a lower percentage of mucus in their guts than endogeic earthworms which seems to correlate with the amount of carbon ingested with soil (Trigo *et al.* 1999). Epigeic and anecic earthworms have a more carbon rich diet than endogeic earthworms do, and thus, the more carbon rich the ingested substrate is the less mucus is secreted to the gut (Trigo *et al.* 1999).

The earthworm gut is characterized by a high water content, a neutral pH, a high content of mucus-derived saccharides, and anoxia (Barois and Lavelle 1986, Brown 1995, Trigo *et al.* 1999, Horn *et al.* 2003b, Drake and Horn 2007), and thus constitutes an ideal habitat for soil-derived anaerobes. Microorganisms in the gut derive from substrate that the earthworm feeds on (Bassalik 1913, Karsten and Drake 1997, Ihssen *et al.* 2003, Horn *et al*. 2006b, Wüst *et al.* 2009b, Contreras 1980, Drake and Horn 2007). Different earthworm species affect the ingested microbial community differently (Brown 1995). For example, cultivable cell numbers increase during gut passage in *Lumbricus rubellus* but decrease in *Aporrectodea caliginosa* (Kristufek *et al.* 1992, Brown 1995). The ratio of microorganisms that grow under obligate anoxic conditions to those growing under oxic conditions was higher with gut contents of earthworms than with soil (Karsten and Drake 1995). In general, the number of cultivable microorganisms in the gut is higher than in preingested soil and anaerobes can be 4-4,000 times more abundant in the gut than in the soil (Drake and Horn 2007).

Living earthworms emit denitrification-derived N_2, the greenhouse gas nitrous oxide (N_2O), and fermentation-derived H_2 (Karsten and Drake 1997, Horn *et al*. 2006a, Wüst *et al.* 2009b). H_2 and N_2O are present in the O_2-free gut center of the earthworm *L. terrestris* (Wüst *et al*. 2009b). N_2 and N_2O are produced by denitrifiers such as *Bradyrhizobium*, *Sinorhizobium*,

and *Pseudomonas* in the earthworm gut (Horn *et al*. 2006b), and H_2 from fermenters such as glucose-fermenting taxa belonging to *Clostridiaceae* and *Enterobacteriaceae* (Wüst *et al.* 2011). Molecular and cultivation-dependent analysis indicated that denitrifiers and fermenters derive from ingested soil and become activated in the earthworm gut (Karsten and Drake 1997, Ihssen *et al.* 2003, Horn *et al*. 2006b, Wüst *et al.* 2009b). N_2O-producing microorganisms were most active in the crop, gizzard, and hindgut whereas H_2-producing fermenters were most active in foregut and hindgut (Wüst *et al*. 2009b). Denitrifiers in the earthworm gut might utilize sugars and organic acids that originate from hydrolysis and fermentation of mucus-derived saccharides. Slurries with gut contents consumed saccharides more rapidly than slurries with soil (Karsten and Drake 1995), illustrating that the earthworm gut constitutes an ideal habitat for anaerobes such as fermenters and denitrifiers. Besides fermenters and denitrifiers (Drake *et al.* 2009, Palmer *et al.* 2010), acetogens and methanogens are examples of anaerobes that are important in wetland soils (Drake *et al.* 2009) but generally appear to be rather unimportant in the earthworm gut (Hornor and Mitchell 1981, Karsten and Drake 1997). Exceptions might be found in the earthworms *E. eugeniae*, *P. corethrurus*, and *Rhinodrilus alatus* that have recently been discovered to emit CH_4 *in vivo* (Depkat-Jakob *et al.* 2012).

3.5 Anaerobic food webs drive the emission of CH_4

Anoxic CH_4-emitting habitats have in common that a trophically linked microbial community degrades complex organic matter to intermediates that terminally drive methanogenesis if CO_2 is the major terminal electron acceptor, collectively called ‘intermediary ecosystem metabolism’ (Figure 3) (Zinder 1993, Glissmann and Conrad 2000, Kotsyurbenko 2005, Drake *et al.* 2009, Brune 2014). Mire soils, the rhizosphere of wetland plants, and the earthworm gut are examples of anoxic habitats with complex microbial food webs and communities (Kraigher *et al.* 2006, Lu *et al*. 2006, Drake and Horn 2007, Drake *et al*. 2009, Wu *et al.* 2009, Dedysh 2011, Wüst *et al.* 2011, Lin *et al.* 2014a, Lin *et al.* 2014b, Bertani *et al.* 2016).

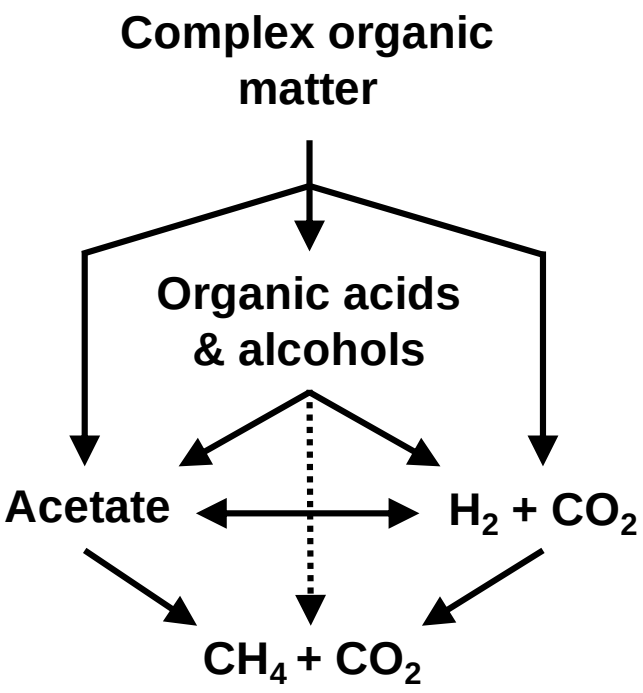

Figure 3: Hypothetical model of the intermediary ecosystem metabolism in CH_4-emitting habitats if CO_2 is the main terminal electron acceptor.

The dashed line indicates that some of the intermediates such as formate and methanol can be used by methanogens. Figure was modified from Zehnder (1978), McInerney and Bryant (1981), and Drake *et al.* (2009).

Plant-derived biopolymers are the major input of carbon into mire soils, rhizosphere of wetland plants, and also a source of carbon for microorganisms in the gut of litter-feeding earthworms (Zinder 1993, Watanabe *et al.* 1999, Brown *et al.* 2000, Ding *et al.* 2002). Lignocellulose constitutes a main component of plant-derived biomass and can account for 50-80 % of total plant biomass (Ahmed *et al.* 2001). Lignocellulose consists of cellulose, hemicellulose, and lignin (Ahmed *et al.* 2001). Cellulose and hemicellulose are polymers that consist of sugars (Kokorevics *et al.* 1997, Ahmed *et al.* 2001). Cellulose consists of glucose only whereas hemicellulose consists mostly of xylose, other sugars, and only partially of glucose (Malburg *et al.* 1992, Kokorevics *et al.* 1997, Ahmed *et al.* 2001, Lynd *et al.* 2002). Microorganisms excrete exoenzymes that hydrolyze polymers to mono- and disaccharides and subsequently ferment those (Kang *et al.* 2004, Kotsyurbenko 2005, Drake *et al.* 2009). The major input of carbon for microorganisms in the earthworm gut derives from mucus (Lavelle *et al.* 1995, Martin *et al.* 1987, Trigo *et al.* 1999, Drake and Horn 2007). Gut contents may contain

up to 80 % worm-derived mucus (Trigo *et al.* 1999) and enzymes that degrade oligosaccharides and polysaccharides (Zhang *et al.* 1993). Some of those enzymes derive from ingested microorganisms (Zhang *et al.* 1993). The highest amount of those enzymes was detected in foregut and midgut where the amount of mucus is the highest (Zhang *et al.* 1993, Trigo *et al.* 1999), indicating that those enzymes may contribute to the degradation of mucus. Hydrolyzed mucus consists of diverse mono- and disaccharides including glucose (Rahemtulla and Løvtrup 1975, Wüst *et al.* 2009b).

Fermentation products indicative for mixed acid, propionate, and butyrate fermentation have been detected in gut contents of *L. terrestris* and moderate acidic mire soil (Hamberger *et al.* 2008, Drake *et al.* 2009, Wüst *et al.* 2009b). Additionally, fermentation products indicative of amino acid and lactate fermentation have been detected in gut contents of *L. terrestris* (Wüst *et al.* 2009b). With soil from a moderate-acid mire, cellulose was degraded to propionate, acetate and CO_2 and *Porphyromonadaceae*, *Acidobacteriaceae* and *Ruminococcaceae* were identified as active fermenters that utilize cellulose directly or utilize cellulose-derived cellobiose or glucose indirectly (Schmidt *et al.* 2015). In taiga pond sediments, cellulose is fermented to acetate, propionate, iso-valerate, and H_2 (Kotsyurbenko 2005). In soil of a moderate-acidic mire, glucose and xylose are fermented to acetate, butyrate, propionate, formate, CO_2 and H_2 (Hamberger *et al.* 2008). *Acidaminococcaceae*, *Actinomycetales*, *Aeromonadaceae*, *Clostridiaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* were identified as taxa being involved in the degradation of glucose and xylose (Hamberger *et al.* 2008). *Clostridium intestinale* was isolated from roots of a wetland plant and grows by fermentation under anoxic conditions (Gößner *et al.* 2006). *C. intestinale* utilizes cellobiose, glucose, fructose, and N-acetylglucosamine and produces acetate, butyrate, lactate, formate and H_2 as fermentation products (Gößner *et al.* 2006). Taxa potentially capable of fermentation were detected in the rhizosphere of rice plants (e.g., *Clostridia*, *Acidobacteria*, *Bacteroidetes* [Lu *et al.* 2006]). With gut contents of *L. terrestris*, the fermentation of glucose yielded acetate, butyrate, formate, lactate, propionate, succinate, ethanol, H_2 and CO_2, and *Enterobacteriaceae* and *Clostridiaceae* were identified as active glucose-utilizing fermenters (Wüst *et al.* 2011).

Products of primary fermentation such as ethanol, butyrate and propionate can be utilized by secondary fermenters that form H_2, CO_2, formate and acetate (McInerney and Bryant 1981, Schink 1997). Secondary fermenters are most often trophically linked with an H_2- or acetate-utilizing microorganism that makes the dissimilation of ethanol, butyrate, and propionate thermodynamically feasible under anoxic conditions (Schink 1997). Such a partner can be a methanogen or an acetogen (Bryant *et al.* 1977, Schink 1997). Thus, secondary fermenters link primary fermentation with acetogenesis and methanogenesis (Jackson *et al.* 1999, Lengeler *et al.* 1999).

Acetogens form acetate that subsequently fuels methanogenesis (Balch *et al.* 1979, Drake *et al.* 2006, Hädrich *et al.* 2012). Acetogens in mire soils utilize H_2-CO_2, formate, methanol, and potentially many other substrates including sugars (Wüst *et al.* 2009a, Hädrich *et al.* 2012). Acetogens in paddy soils and attached to rice roots utilize H_2-CO_2 and potentially other substrates, and have been associated with species of *Clostridium* and *Sporomusa* (Conrad and Klose 1999, Liu and Conrad 2011). Acetogens active in those habitats are mostly unknown (Drake *et al.* 2009, Hädrich *et al.* 2012). Two acetogens, *Sporomusa rhizae* and *Terrisporobacter glycolicus*, have been isolated from roots of wetland plants (Küsel *et al.* 2001, Gößner *et al.* 2006). Both acetogens grow on H_2-CO_2, formate, and lactate (Küsel *et al.* 2001, Gößner *et al.* 2006). Acetogenesis in the earthworm gut is hitherto thought to be unimportant (Drake *et al.* 2009).

Methanogenesis in mire soils is studied well. Mire-derived methanogens utilize H_2-CO_2, formate, acetate, and methanol (Williams and Crawford 1984, Horn *et al.* 2003a, Cadillo-Quiroz *et al.* 2006, Wüst *et al.* 2009a). *Methanobacteriaceae*, *Methanocellaceae*, *Methanomicrobiaceae*, *Methanoregulaceae*, "*Methanosaetaceae*", and *Methanosarcinaceae* have been detected in mire soils (Horn *et al.* 2003a, Juottonen *et al.* 2005, Cadillo-Quiroz *et al.* 2006, Hamberger *et al*. 2008, Putkinen *et al.* 2009, Wüst *et al.* 2009a, Lin *et al.* 2014a). Methanogens that are capable to grow on H_2-CO_2 and acetate were detected in rhizospheric soil and attached to roots of rice plants (i.e., *Methanomicrobiaceae*, *Methanobacteriaceae*,

Methanosarcinaceae, "*Methanosaetaceae*", *Methanocellaceae* [Chin *et al.* 2004, Lu and Conrad 2005]). Methanogenesis in the earthworm gut is hitherto thought to be unimportant (Drake *et al.* 2009) but *in vivo* emission of CH_4 from earthworms has recently been discovered (Depkat-Jakob *et al.* 2012).

Collectively those studies indicate that similar processes occur in diverse anoxic habitats and those processes seem to be linked to different taxa, indicating a functional redundancy of microorganisms. The fermentation of complex organic matter and the production of CH_4 have been well studied in anoxic habitats but intermediary trophic links and microorganisms involved in those processes are less studied and mostly conceptualized (Karsten and Drake 1995, Kotsyurbenko *et al.* 1996, Glissmann and Conrad 2000, Bräuer *et al.* 2004, Drake *et al.* 2009, Wüst *et al.* 2009a, Schmidt *et al.* 2015).

3.6 Fermentation

Fermentation is widespread within *Bacteria*, *Archaea*, and Eukaryotes (Lengeler *et al.* 1999). Species related to *Clostridiaceae* (Wiegel 2009), *Acidobacteriaceae* (Pankratov *et al.* 2012), *Planctomycetaceae* (Kulichevskaya *et al.* 2007), *Veillonellaceae* (Rainey 2009b), and many other bacterial taxa are capable of fermentation.

Table 1: Cultivable cell numbers of fermenters in paddy and fen soil.

Supplemental substrate	Cultivable cell number per g_{DW}[a]	Habitat	Reference
Xylan	0.4 – 9.4 x 10^8	Paddy soil	Chin *et al.* 1999
Cellobiose	0.1 – 20 x 10^6	Paddy soil	Chin *et al.* 1999
Sugars mixture	0.8 – 13 x 10^7	Paddy soil	Chin *et al.* 1999
Glucose	0.1 – 25 x 10^6	Paddy soil	Chin *et al.* 1999
Glucose	0.01 – 10 x 10^7	Fen soil	Wüst *et al.* 2009a
Xylose	0.8 – 50 x 10^6	Fen soil	Wüst *et al.* 2009a

[a] Cell numbers may contain a minor part of non-fermenters growing on supplemental substrates.

Fermenters were isolated from divers habitats such as swamp (Su *et al.* 2014), anaerobic sludge (Qiu *et al.* 2014), digester sludge (Alves *et al.* 2013), rumen (Sun *et al.* 2015),

and wetland soil (Kulichevskaya *et al.* 2014b). Fermenters in wetland soils can account for approximately 0.8 x 10^6 to 9.4 x 10^9 cultivable fermenters g_{DW}^{-1} (Table 1)(Chin *et al.* 1999, Wüst *et al.* 2009a).

Various fermentation pathways are known (Müller 2001). Most are named after the major fermentation end product (Lengeler *et al.* 1999, Müller 2001). Sugars, organic acids, amino acids, polyols, and purine may serve as substrates (Linden 1988, Lengeler *et al.* 1999, Müller 2001). Glucose can be fermented predominantly to (a) lactate (lactic acid fermentation), (b) propionate (propionic acid fermentation), (c) butyrate (butyrate fermentation), (d) a mixture of acids (mixed acid fermentation), or (e) ethanol (ethanol fermentation) (Table 2). Propionate, butyrate, and ethanol fermentation are thermodynamically more favorable than homolactic and heterolactic fermentation (Table 2) (Linden 1988, Lengeler *et al.* 1999, and Müller 2001).

Table 2: Representative fermentation pathways of glucose.[a]

Fermentation pathway	Overall stoichiometry for the fermentation of glucose	Standard change in Gibbs free energy $\Delta G°'$ (kJ mol^{-1})[b]
Homolactic fermentation	$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOO^- + 2\ H^+$	-198
Heterolactic fermentation	$C_6H_{12}O_6 \rightarrow CH_3CHOHCOO^- + H^+ + CH_3CH_2OH + CO_2$	-211
Propionate fermentation	$3\ C_6H_{12}O_6 \rightarrow 4\ CH_3CH_2COO^- + 2\ CH_3COO^- + 6\ H^+ + 2\ CO_2 + 2\ H_2O$	-934
Butyrate fermentation	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COO^- + H^+ + 2\ CO_2 + 2\ H_2$	-255
Mixed acid fermentation	$C_6H_{12}O_6 \rightarrow COO^-CH_2CH_2COO^- + CH_3CHOHCOO^- + CH_3COO^- + HCOO^- + CH_3CH_2OH + CO_2 + H_2$ (non stoichiometric)	n.a.
Ethanol fermentation	$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$	-236

[a] Data derived from Linden (1988), Lengeler *et al.* (1999), and Müller (2001). Legend: $C_6H_{12}O_6$, glucose; $CH_3CHOHCOO^-$, lactate$^-$; $CH_3CH_2CH_2COO^-$, butyrate$^-$; $COO^-CH_2CH_2COO^-$, succinate^{2-}; CH_3COO^-, acetate$^-$; $HCOO^-$, formate$^-$; CH_3CH_2OH, ethanol; CO_2, carbon dioxide; H_2, molecular hydrogen; H^+, proton; n.a., not applicable.

[b] Standard changes in Gibbs free energy are given for the complete reaction and derived from Lengeler *et al.* (1999).

An organic compound serves as electron donor and electron acceptor during fermentation (Müller 2001, Cocaign-Bousquet *et al.* 2002, Madigan and Martinko 2006). An

intermediate is formed during oxidation of the organic compound and serves as electron acceptor (Müller 2001). Pyruvate can be an intermediate and is formed by glycolysis (Lengeler *et al.* 1999, Müller 2001, Cocaign-Bousquet *et al.* 2002). The organic compound is partly oxidized and only a part of the energy in this compound can be conserved in ATP (Müller 2001, Cocaign-Bousquet *et al.* 2002). Most of the energy is conserved during glycolysis and is used to synthesize ATP by substrate level phosphorylation (Romano and Conway 1996, Lengeler *et al.* 1999, Madigan and Martinko 2006). Additional ATP can be conserved by a sodium or proton gradient and electron-transport-coupled phosphorylation in some cases (Müller 2001), for example during citrate fermentation with the help of a Na^+-translocating decarboxylase (Dimroth 1997). Less than one mol and up to four moles of ATP can be conserved from one mol of substrate depending on the substrate and fermentation pathway used (Müller 2001).

3.7 Formation of H_2 by the formate-hydrogenlyase complex

Formate is a common product of mixed acid fermentation (Linden 1988, Lengeler *et al.* 1999, Müller 2001). It is either excreted, decomposed to CO_2 and H_2, or utilized as source of electrons to reduce nitrate or fumarate (Peck and Gest 1956, Rossmann *et al.* 1991, Sawers 1994, Leonhartsberger *et al.* 2002). The decomposition of formate to H_2 and CO_2 under anoxic conditions counteracts acidification of the medium and is catalyzed by enzymes such as the formate-hydrogenlyase (FHL) complex and is in some cases coupled to the translocation of protons and potentially the conservation of energy (Stephenson and Stickland 1932, Andrews *et al.* 1997, Bagramyan *et al.* 2002, Graentzdoerffer *et al.* 2003, Trchounian and Sawers 2014). The O_2-sensitive FHL complex consists of a formate dehydrogenase and a hydrogenase (Figure 4) (Peck and Gest 1956, Axley *et al.* 1990).

The presence of intracellular formate is required for the expression of genes encoding the FHL complex (Rossmann *et al.* 1991). Formate dehydrogenase transfers electrons from formate to a hydrogenase (Peck and Gest 1956, Graentzdoerffer *et al.* 2003). The hydrogenase produces H_2 from protons and formate-derived electrons (Sauter *et al.* 1992, Graentzdoerffer *et al.* 2003). *Escherichia coli* harbors two FHL complexes consisting of a

formate dehydrogenase that can be either coupled to a membrane-bound [NiFe]-hydrogenase (Böhm *et al.* 1990, Sauter *et al.* 1992) or a membrane-bound and potentially energy-dependent [NiFe]-hydrogenase (Andrews *et al.* 1997, Bagramyan *et al.* 2002).

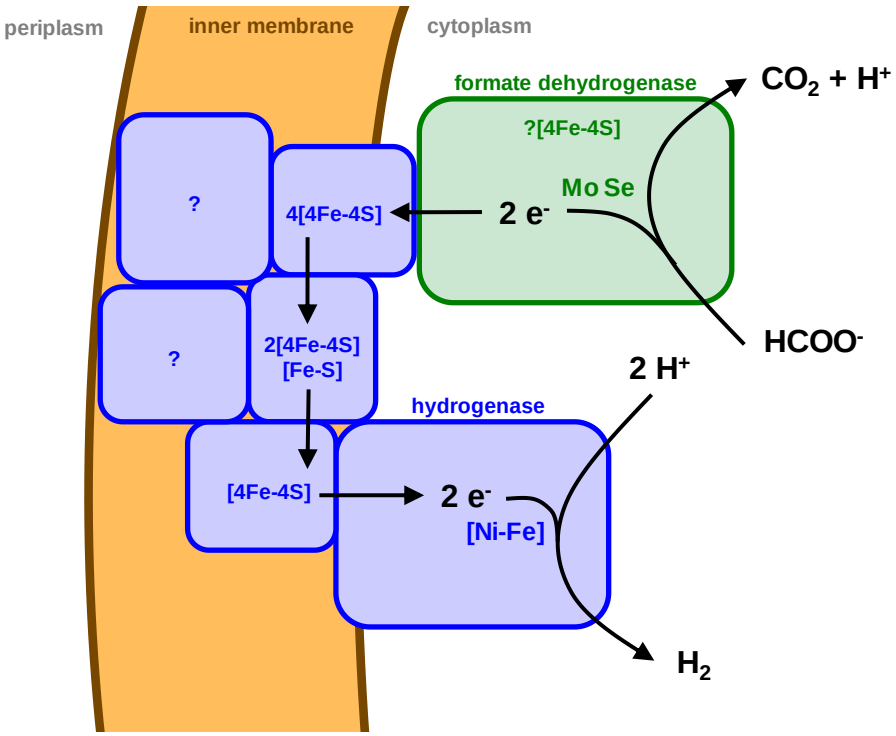

Figure 4: Generalized flow of formate-derived electrons within an FHL complex of *E. coli*.

Figure was created based on observations made by Sauter *et al.* (1992), Andrews *et al.* (1997), and Sawers (1994). Metal clusters are indicated. Legend: blue, subunits of hydrogenase with a Ni-Fe active site of the large subunit; green, formate dehydrogenase; $HCOO^-$, formate$^-$; CO_2, carbon dioxide; H^+, proton; e^-, electron.

Methanogens such as *Methanobacterium formicicum* contain an FHL complex that can form H_2 and CO_2 from high millimolar concentrations of formate or reverse the reaction and form formate from high concentrations of H_2 and CO_2 (Wu *et al.* 1993).

3.8 Acetogenesis

Acetogens produce approximately 10^{13} kg acetate per year accounting for about 10 % of the global acetate production in terrestrial habitats (Wood and Ljungdahl 1991). Acetogens are known to be strict anaerobes but some species show a certain extent of tolerance to O_2 (Drake *et al.* 2008, Küsel *et al.* 2001). Most acetogens belong to the *Bacteria* but also two species of *Archaea* are capable of acetate production via acetogenesis (Rother and Metcalf

2004, Henstra *et al.* 2007, Drake *et al.* 2008). Over 100 different bacterial species are known which belong to the genera *Acetitomaculum*, *Acetoanaerobium*, *Acetobacterium*, *Acetohalobium*, *Acetonema*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Caloramator*, *Clostridium*, *Eubacterium*, *Holophaga*, *Marvinbryantia*, *Moorella*, *Natroniella*, *Natronincola*, *Oxobacter*, *Ruminococcus*, *Sporomusa*, *Syntrophococcus*, *Terrisporobacter*, *Thermacetogenium*, *Thermoanaerobacter*, *Tindallia*, and *Treponema* (Drake *et al.* 2008, Liu *et al.* 2008, Wolin *et al.* 2008, Allen *et al.* 2010, Gerritsen *et al.* 2014). Most acetogens belong to the class *Clostridia* (Drake *et al.* 2008). *Archaeoglobus fulgidus* and *Methanosarcina acetivorans* belong to the *Archaea* and are capable to produce acetate from carbon monoxide (CO) under laboratory conditions (Rother and Metcalf 2004, Henstra *et al.* 2007).

Table 3: Cultivable cell numbers of acetogens in different habitats.

Substrate converted to acetate	Cultivable cell number per (a) g_{DW} or (b) g_{FW}	Habitat	Reference
H_2-CO_2	(a) 3.2 – 70 x 10^1	Conifer litter	Reith *et al.* 2002
	(a) 1.9 – 42 x 10^4	Leaf litter	Reith *et al.* 2002
	(a) 1.8 – 44 x 10^3	Forest soil	Schnurr-Pütz *et al.* 2006
	(b) 0.3 – 70 x 10^5	Rhizosphere sediment	Küsel *et al.* 1999
	(b) 1.9 – 42 x 10^3	Unvegetated sediment	Küsel *et al.* 1999
	(b) 3.1 – 36 x10^5	Rumen, lamb	Doré *et al.* 1995
	(b) 0.03 – 3100 x 10^5	Feces, human	Doré *et al.* 1995
	(b) 1.4 – 3.1 x 10^4	Digester sludge	Doré *et al.* 1995
	(b) 0.9 – 200 x 10^4	Ditch sediment	Harriott and Frazer 1997
Vanillate	(a) 3.2 – 70 x 10^1	Conifer litter	Reith *et al.* 2002
	(a) 0.5 – 11 x 10^2	Leaf litter	Reith *et al.* 2002
	(a) 0.1 – 44 x 10^2	Forest soil	Schnurr-Pütz *et al.* 2006
	(b) 1.9 – 42 x 10^4	Rhizosphere sediment	Küsel *et al.* 1999
	(b) 0.5 – 11 x 10^2	Unvegetated sediment	Küsel *et al.* 1999
	(b) 0.7 – 27 x 10^4	Ditch sediment	Harriott and Frazer 1997
Ethanol	(a) 1.1 – 5.0 x 10^2	Conifer litter	Reith *et al.* 2002
	(a) 0.5 – 11 x 10^3	Leaf litter	Reith *et al.* 2002

Acetogens are widely distributed (Drake *et al.* 2006) and have been isolated from diverse habitats such as the termite gut (Kane *et al.* 1991), human gut (Doré *et al.* 1995), oxic soil (Gößner *et al.* 1999), rhizosphere (Küsel *et al.* 1999), hypersaline water (Ollivier *et al.* 1994), deep subsurface sediments (Liu and Sulfita 1993), or roots of wetland plants (Küsel *et al.* 2001). Cultivable acetogens can account for approximately 3.2×10^1 to 4.2×10^5 H_2-utilizing acetogens g_{DW}^{-1} (Reith *et al.* 2002), 3.2×10^1 to 4.4×10^3 vanillate-utilizing acetogens g_{DW}^{-1} (Reith *et al.* 2002, Schnurr-Pütz *et al.* 2006), 1.1×10^2 to 1.1×10^4 ethanol-utilizing acetogens g_{DW}^{-1} (Reith *et al.* 2002) (Table 3).

Acetogens have a broad substrate range and can grow on saccharides, organic acids, alcohols, CO, and H_2 (Drake *et al.* 2008) (Table 4). The calculated standard changes in Gibbs free energy ($\Delta G°'$) illustrate that acetogens that grow on glucose conserve more energy than acetogens that grow on formate or H_2-CO_2 (Table 4). Consequently, an acetogen that dissimilates glucose may assimilate more carbon, produce more biomass, and grow faster than as acetogen that grows on formate or H_2-CO_2.

Table 4: Representative growth-supportive substrates of acetogens.

Substrate[a]	Overall stoichiometry for the production of acetate[a]	Standard change in Gibbs free energy $\Delta G°'$ (kJ mol^{-1})[b]
Cellobiose	$C_{12}H_{22}O_{11} + H_2O \rightarrow 6\ CH_3COOH$	-675
Glucose	$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$	-310
Formic acid	$4\ HCOOH \rightarrow CH_3COOH + 2\ CO_2 + 2\ H_2O$	-109
Ethanol	$2\ CH_3CH_2OH + 2\ CO_2 \rightarrow 3\ CH_3COOH$	-75
Methanol	$4\ CH_3OH + 2\ CO_2 \rightarrow 3\ CH_3COOH + 2\ H_2O$	-211
CO	$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2$	-175
H_2-CO_2	$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$	-95
H_2-CO	$2\ H_2 + 2\ CO \rightarrow CH_3COOH$	-135

[a] Data derived from Drake (1994). Legend: $C_{12}H_{22}O_{12}$, cellobiose; $C_6H_{12}O_6$, glucose; HCOOH, formic acid; CH_3COOH, acetic acid; CH_3CH_2OH, ethanol; CH_3OH, methanol; CO, carbon monoxide; CO_2, carbon dioxide; H_2, molecular hydrogen, H_2O, water.

[b] Standard changes in Gibbs free energy were calculated based on the Gibbs free energy of formation and are given for the complete reaction (Thauer *et al.* 1977, Conrad and Wetter 1990, Berg *et al.* 2003).

Acetogens use CO_2 as terminal electron acceptor and produce acetate via the acetyl-CoA "Wood/Ljungdahl" pathway (Drake *et al.* 2008) (Figure 5). The acetyl-CoA pathway is linear and CO_2 is not bound to intermediates of the pathway (Drake *et al.* 2008). In contrast, pyruvate is carboxylated with CO_2 to form oxaloacetate in the citric acid cycle (Berg *et al.* 2003). Because of the simplicity and linearity of the acetyl-CoA pathway, it is speculated that this pathway was used for energy conservation in first free-living cells rather than complex cycles such as the citric acid cycle (Fuchs 1986, Sousa *et al.* 2013, Nitschke and Russell 2013).

The acetyl-CoA pathway is composed of two branches named methyl and carbonyl branch (Figure 5) (Drake *et al.* 2006). CO_2 is fixed in both branches and is reduced to a methyl group in the methyl branch or to a carbonyl group in the carbonyl branch (Drake *et al.* 2006, Ragsdale and Pierce 2008). In the methyl branch, formate dehydrogenase reduces CO_2 to formate (Drake *et al.* 2006, Ragsdale and Pierce 2008). Formate is activated by formyltetrahydrofolate synthetase with an adenosine triphosphate (ATP) (Drake *et al.* 2006, Ragsdale and Pierce 2008). Formyltetrahydrofolate synthetase is encode by *fhs* that can be used as structural gene marker in molecular analysis to study acetogens (Leaphart and Lovell 2001). The formyl group is bound to tetrahydrofolate and is further reduced to a methyl group (Drake *et al.* 2006, Ragsdale and Pierce 2008). A corrinoid protein transfers the methyl group from tetrahydrofolate to CO dehydrogenase/acetyl-CoA synthase complex (CODH/ACS complex) (Ragsdale and Pierce 2008). In the carbonyl branch, the CO dehydrogenase function of the CODH/ACS complex reduces CO_2 to a carbonyl group (Drake *et al.* 2006, Ragsdale and Pierce 2008). CODH/ACS complex catalyzes the formation of acetyl-CoA from coenzyme A, the methyl group, and the carbonyl group (Drake *et al.* 2006, Ragsdale and Pierce 2008). Acetyl-CoA is either used for assimilation or for energy conservation via substrate-level phosphorylation and formation of acetate (Drake *et al.* 2006, Ragsdale and Pierce 2008). Acetate kinase catalyzes the reaction from acetyl-phosphate to acetate and at the same time forms ATP by substrate-level phosphorylation (Drake *et al.* 2006, Ragsdale and

Pierce 2008). Additional ATP is conserved from a sodium motive force and electron-transport-coupled phosphorylation that involves the Rnf complex (Poehlein *et al.* 2012).

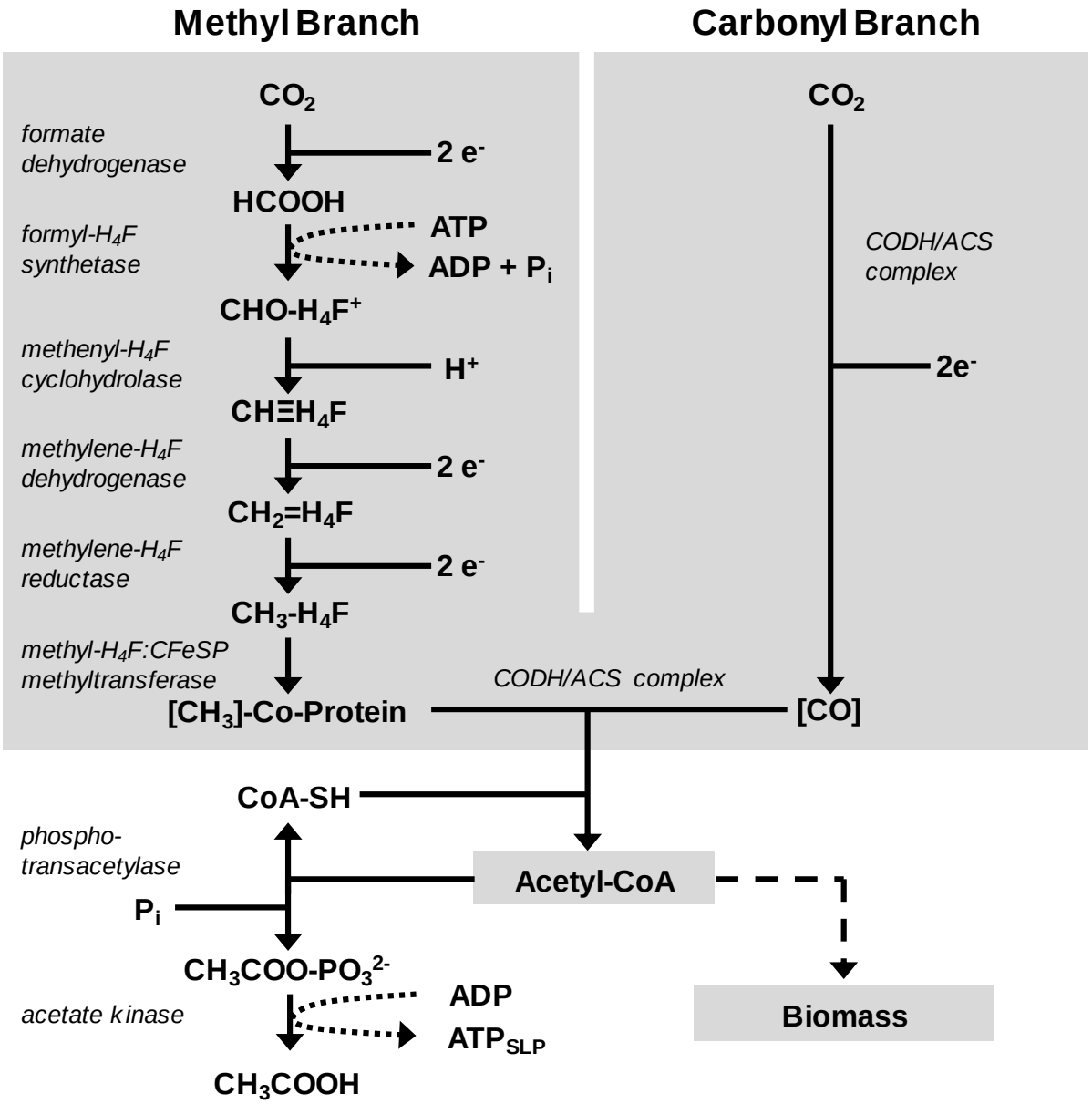

Figure 5: The acetyl-CoA "Wood/Ljungdahl" pathway.

Figure was modified from Müller *et al.* (2004) and Drake *et al.* (2008). Legend: CH_3COOH, acetic acid; $CH_3COO\text{-}PO_3^{2-}$, acetyl phosphate; HCOOH, formic acid; CO_2, carbon dioxide; $CHO\text{-}H_4F^+$, formyltetrahydrofolate; $CH\Xi H_4F$, methenyltetrahydrofolate; $CH_2{=}H_4F$, methylenetetrahydrofolate; $CH_3\text{-}H_4F$, methyltetrahydrofolate; H_4F, tetrahydrofolate; $[CH_3]$, methyl group; [CO], carbonyl group; CODH/ACS complex, CO dehydrogenase/acetyl-CoA synthase complex; CoA, coenzyme A; P_i, inorganic phosphate; e^-, electron; Co-protein, corrinoid protein; ADP, adenosine diphosphate; ATP, adenosine triphosphate; SLP, substrate-level phosphorylation.

Acetogens that grow on sugars conserve energy from glycolysis, the oxidation of pyruvate to acetate, and the acetyl-CoA pathway (Drake *et al.* 2006). For example, three

molecules of acetate are produced from one molecule of glucose (Drake *et al.* 2006). Two molecules of acetate derive from glycolysis and the oxidation of pyruvate, and one molecule derives from the acetyl-CoA pathway (Drake *et al.* 2006). Two molecules of CO_2 are produced during the oxidation of pyruvate (Drake *et al.* 2006). In theory, it looks as if the produced CO_2 could be reduced in the acetyl-CoA pathway and acetogens would be independent of additional CO_2. Interestingly, growth of acetogens on sugars can be impaired if grown without supplemental CO_2 (Andreesen *et al.* 1970, O'Brian and Ljungdahl 1972, Braun and Gottschalk 1981). Studies with ^{14}C demonstrated that approximately one-third of glucose-derived carbon is recovered in CO_2 and the other two-thirds are recovered in acetate, indicating that endogenous CO_2 is used to form one acetate (Drake *et al*. 2006).

3.9 Methanogenesis

Methanogens produce approximately one billion tons of CH_4 per year on a global scale (Thauer 1998). All methanogens are strict anaerobes and belong to the *Archaea* (phylum *Euryarchaeota*) (Balch *et al.* 1979, Thauer 1998, LPSN, http://www.bacterio.net). Approximately 200 different methanogenic species are known, which belong to the genera *Halomethanococcus*, *Methanimicrococcus*, *Methanobacterium*, *Methanobrevibacter*, *Methanocalculus*, *Methanocaldococcus*, *Methanocella*, *Methanococcoides*, *Methanococcus*, *Methanocorpusculum*, *Methanoculleus*, *Methanofollis*, *Methanogenium*, *Methanohalobium*, *Methanohalophilus*, *Methanolacinia*, *Methanolinea*, *Methanolobus*, *Methanomassiliicoccus*, *Methanomicrobium*, *Methanomethylovorans*, *Methanoplanus*, *Methanopyrus*, *Methanoregula*, *Methanosaeta*, *Methanosalsum*, *Methanosarcina*, *Methanosphaera*, *Methanosphaerula*, *Methanospirillum*, *Methanothermobacter*, *Methanothermococcus*, *Methanothermus*, *Methanothrix*, *Methanotorris*, and *Methermicoccus* (LPSN, http://www.bacterio.net). Most species belong to the family *Methanobacteriaceae* (LPSN, http://www.bacterio.net).

Table 5: Cultivable cell numbers of methanogens in different habitats.

Substrate converted to CH_4	Cultivable cell number per (a) g_{DW} or (b) g_{FW}	Habitat	Reference
Fermentation products	(a) 0.2 – 20 x 10^5	Fen soil	Wüst *et al.* 2009a
	(a) 0.2 – 200 x 10^2	Forest soil	Schnurr-Pütz *et al.* 2006
H_2-CO_2	(a) 0.6 – 10 x 10^7	Bog soil	Horn *et al.* 2003a
Acetate	(a) 0.2 – 5.4 X 10^5	Paddy soil	Mizukami *et al.* 2006
Methanol, H_2-CO_2	(b) 9 – 66 x 10^6	Permafrost soil	Morozova *et al.* 2007

Methanogens are widely distributed and have been isolated from diverse habitats such as human feces (Dridi *et al.* 2012), paddy soil (Dianou *et al.* 2001), anaerobic digester (Zellner *et al.* 1998), oil field (Cheng *et al.* 2008), deep sea marine sediments (Kurr *et al.* 1991, Mikucki *et al.* 2003), Arctic permafrost sediments (Shcherbakova *et al.* 2011), or peatlands (Bräuer *et al.* 2011, Cadillo-Quiroz *et al.* 2014).

Table 6: *mcrA* copy numbers of methanogens in different habitats.

Substrate converted to CH_4	*mcrA* copy numbers per (a) g_{DW} or (b) g_{FW}	Habitat	Reference
Fermentation products	(a) 2.1 x 10^9	Biogas reactor	Kampmann *et al.* 2012
Unknown[a]	(a) 0.1 – 1.2 x 10^8	River sediment	Zeleke *et al.* 2013
	(b) 10^4 – 10^7	Marine sediment	Schippers *et al.* 2012
	(b) 0.1 – 7.9 x 10^8	Peat	Freitag *et al.* 2010
	(b) 10^6 – 10^9	Wetland soil	Bae *et al.* 2015

[a] The substrate of methanogenesis is not given but CH_4 was likely produced from fermentation products such as acetate, formate, and H_2-CO_2.

Cultivable methanogens can account for approximately 0.2 x 10^2 to 1.0 x 10^8 methanogens g_{DW}^{-1}, and can be more abundant in water-saturated soil than in forest soil (Table 5) (Horn *et al.* 2003a, Schnurr-Pütz *et al.* 2006). *mcrA* gene copy numbers can range from 10^7 to 10^9 g_{DW}^{-1} (Table 6) (Kampmann *et al.* 2012, Zeleke *et al.* 2013).

Table 7: Representative growth-supportive substrates of methanogens.

Substrate[a]	Overall stoichiometry for the production of CH_4[a]	Standard change in Gibbs free energy $\Delta G°'$ (kJ mol^{-1})[b]
Acetic acid	$CH_3COOH \rightarrow CH_4 + CO_2$	-36
Formic acid	$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	-145
Methanol	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	-320
H_2-methanol	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-112
H_2-CO_2	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-131

[a] Data derived from Zinder (1994). Legend: CH_3COOH, acetic acid; HCOOH, formic acid; CH_3OH, methanol; CO_2, carbon dioxide; H_2, molecular hydrogen; CH_4, methane; H_2O, water.
[b] Standard change in Gibbs free energy was calculated based on the Gibbs free energy of formation and is given for the complete reaction (Thauer *et al.* 1977, Conrad and Wetter 1990, Berg *et al.* 2003).

The substrate range of methanogens is restricted (Zinder 1994, Balch *et al.* 1979). Most methanogens grow on acetate, formate, methanol, methylamine, or H_2-CO_2 (Balch *et al.* 1979, Zinder 1994). Some methanogens may additionally use secondary alcohols such as 2-propanol or 2-butanol (Maestrojuán *et al.* 1990, Zellner *et al.* 1998, Dianou *et al.* 2001). Growth on methanol, formate, or H_2-CO_2 is thermodynamically more favorable than growth on acetate (Table 7).

Similar to the acetyl-CoA "Wood/Ljungdahl" pathway, it is speculated that methanogenesis was one of the early pathways that were used for energy conservation in free-living cells (Sousa *et al.* 2013). The formation of CH_4 from formate occurs stepwise (Figure 6) (Thauer 1998). Formate is first converted to H_2 and CO_2 and CO_2 is subsequently reduced to CH_4 with H_2 (Thauer 1998). The formyl group of CO_2 is first bound to methanofuran by formylmethanofuran:H_4MPT formyltransferase and is then transferred to tetrahydro-methanopterin (H_4MPT) (Shima *et al.* 1995, Thauer 1998). The formyl group is subsequently reduced to a methyl group (Thauer 1998). Methyl-H_4MPT:coenzyme M methyltransferase transfers the methyl group from tetrahydromethanopterin to coenzyme M (Thauer 1998). When methanol is used, the methyl group is directly transferred from methanol to coenzyme M by methanol:coenzyme M methyltransferase (Sauer *et al.* 1997).

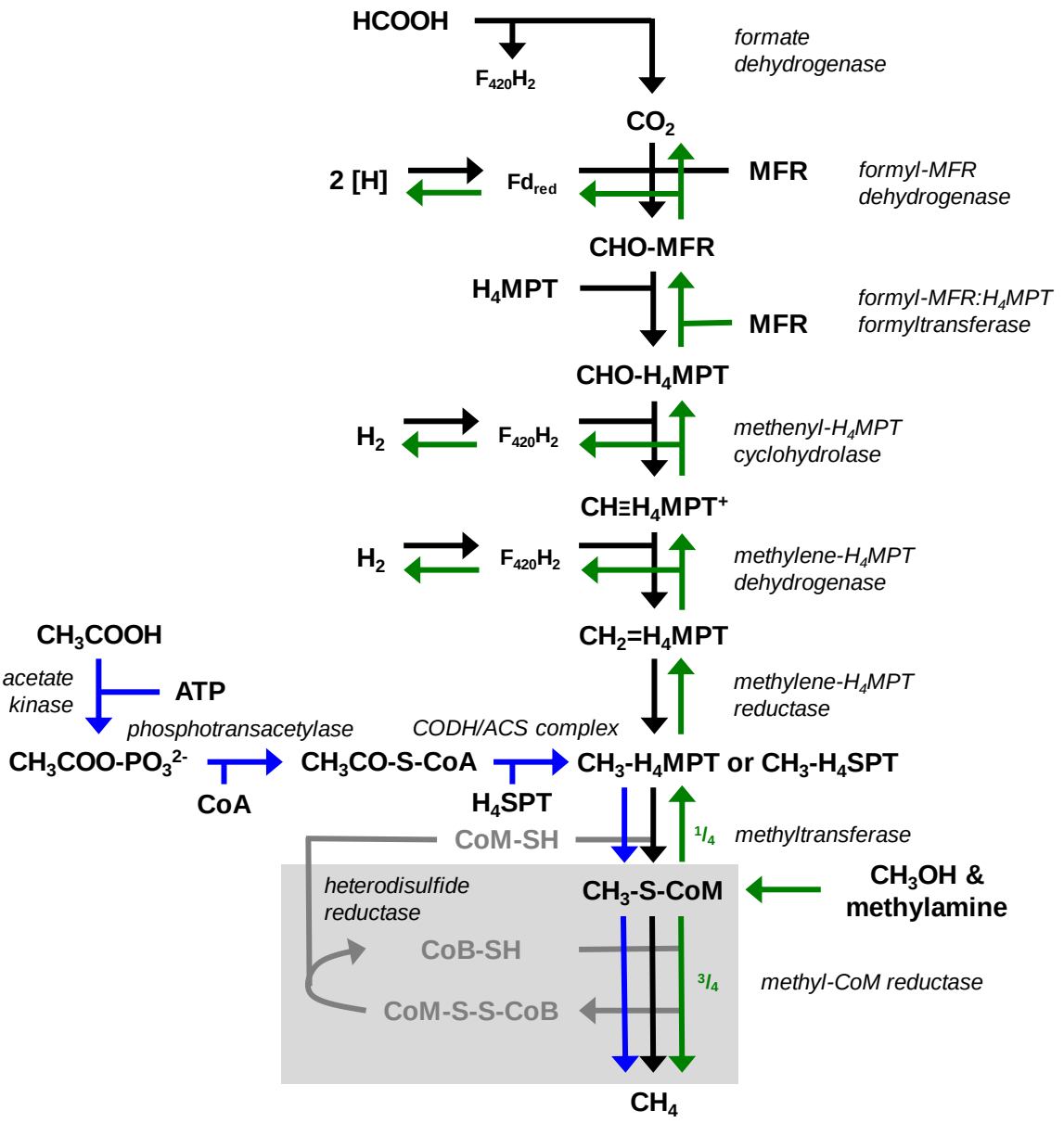

Figure 6: Reactions and enzymes involved in the formation of CH_4 from H_2-CO_2, formic acid, acetic acid, methanol, and methylamine.

Figure was created based on information published in Thauer (1998) and Welte and Deppenmeier (2014). The figure is simplified and does not show all reactants and products of reactions. Legend: CH_3COOH, acetic acid; $CH_3COO\text{-}PO_3^{2-}$, acetyl phosphate; HCOOH, formic acid; CO_2, carbon dioxide; CH_4, methane; H_2, molecular hydrogen; CHO-MFR, formylmethanofuran; CHO-H_4MPT, formyltetrahydromethanopterin; $CH\Xi H_4MPT^+$, methenyltetrahydromethanopterin; CH_2=H_4MPT, methylenetetrahydromethanopterin; CH_3-H_4MPT, methyltetrahydromethanopterin; CH_3-H_4SPT, methyltetrahydrosarcinapterin; CH_3-S-CoM, methyl-coenzyme M; CODH/ACS, carbon monoxide dehydrogenase/ acetyl-CoA synthase; [H], hydrogen atoms; ATP, adenosine triphosphate; CoA, coenzyme A; CoB, coenzyme B; CoM, coenzyme M; CoM-S-S-CoB, heterodisulfide of coenzymes M and B; $F_{420}H_2$, reduced cofactor F_{420}; Fd_{red}, reduced ferredoxin; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; H_4SPT, tetrahydrosarcinapterin; black lines, formic acid and H_2-CO_2 consuming methanogens; blue lines, aceticlastic methanogens; green, methanol and methylamine consuming methanogens; grey box, reactions occurs in all methanogens.

When acetate is used, acetate is bound to coenzyme A and acetyl-CoA is produced by acetate kinase and/or phosphotransacetylase (Latimer and Ferry 1993, Thauer 1998). The methyl group of acetyl-CoA is transferred to tetrahydrosarcinapterin (H_4SPT) by CODH/ACS complex and further transferred to coenzyme M by methyl-H_4SPT:coenzyme M methyltransferase (Fischer *et al.* 1992). Methyl-coenzyme M reductase catalyzes the final reaction and the release of CH_4 from coenzyme M (Shima *et al.* 1997, Bonacker *et al.* 1993). A heterodisulfide is produced from coenzyme M and coenzyme B during this final reaction (Thauer 1998). Methyl-coenzyme M reductase is encoded by *mcrBDCGA* and *mrtBDGA* (Lehmacher and Klenk 1994, Thauer 1998). The α subunit of methyl-coenzyme M reductase is encoded by *mcrA* and *mrtA* and can be used as gene marker for molecular analysis (Springer *et al.* 1995, Lueders *et al.* 2001). The transfer of the methyl group to coenzyme M by a methyltransferase is coupled to the generation of a sodium gradient, and thus, the conservation of energy (Thauer 1998, Thauer *et al.* 2008). The reduction of the heterodisulfide with H_2 is coupled to the generation of a proton gradient (Thauer 1998, Deppenmeier and Müller 2007, Thauer *et al.* 2008). Additional ATP is conserved from a sodium motive force and electron-transport-coupled phosphorylation that involves the Rnf complex (Welte and Deppenmeier 2014).

3.10 Hypothesis and Objectives

CH_4 is one of the most important greenhouse gases in our atmosphere (Stocker *et al.* 2013). A considerable amount of CH_4 is formed from methanogenesis in diverse anoxic habitats (Kotsyurbenko *et al*. 1996, Thauer 1998, Zellner *et al.* 1998, Bräuer *et al.* 2004, Dridi *et al.* 2012, Stocker *et al.* 2013) and wetland plants can mediate the emission of CH_4 from water-satuarated soil by releasing root-derived organic carbon that serve as precursors of CH_4 production (Jones 1998, Ström *et al.* 2003). Most microbiological studies that have investigated the production of CH_4 in anoxic habitats have focused on either a single intermediary process linked to CH_4 or a single habitat (e.g., Kotsyurbenko *et al*. 1996, Bräuer *et al.* 2004, Cadillo-Quiroz *et al.* 2006, Drake *et al.* 2009, Lin *et al.* 2014a, Lin *et al.* 2014b),

and little is known about the potential differences and similarities of anaerobic processes and associated microbial communities driving methanogenesis in contrasting methanogenic food webs (i.e., potential functional redundancy of anaerobes).

Plant roots and earthworms have common impacts on their environment: they (a) influence the microbial community in soil (Lavelle 1986, Jones 1998, Brown *et al.* 2000, Bais *et al.* 2006), (b) supply soil-derived microorganisms with easily available carbon (Martin *et al.* 1987, Uren 2001, Walker *et al.* 2003, Wüst *et al.* 2009b), and (c) increase the number of microorganisms in comparison to bulk soil (Brimecombe *et al.* 2001, Pinton *et al.* 2001, Drake and Horn 2007).

Although fermentation, acetogenesis, and methanogenesis are facilitated by root-containing mire soils (Bräuer *et al.* 2004, Drake *et al.* 2009, Hunger *et al.* 2011a, Hunger *et al.* 2015, Schmidt *et al.* 2016), functional links between active microorganisms and the roots themselves are not well established. Formate can be an important intermediate of the anaerobic food web in mire soils and can reach concentrations of up to 0.65 mM in mire pore water (Küsel *et al.* 2008). It derives from exudates of wetland plant roots (Koelbener *et al.* 2010) or from fermentation of plant-derived polymers (Drake *et al.* 2009), and is a substrate that can be rapidly utilized by mire methanogens (Hunger *et al.* 2011a). Fermenters, acetogens, and methanogens can be associated with roots of wetland plants (Conrad and Klose 1999, Küsel *et al.* 2001, Chin *et al.* 2004, Gößner *et al.* 2006). The availability of root-derived organic carbon, such as sugars, and root-derived methanogenic and acetogenic substrates, such as formate, suggests that those functional groups might also be associated with other wetland plant roots. Despite the conceptualized importance of the wetland root to the production of CH_4, information on specific mechanisms by which the root zone mediates the transformation of plant-derived organic carbon to CH_4 is scant.

The earthworm gut constitutes an ideal habitat for soil-derived anaerobes that grow on mucus which is excreted by the earthworm (Brown *et al.* 2000, Drake and Horn 2007). Studies with different earthworm species showed that fermentation and denitrification are important

microbially mediated processes in the earthworm gut (Karsten and Drake 1997, Horn *et al.* 2006a, Wüst *et al.* 2009b, Wüst *et al.* 2011). Some earthworm species such as *E. eugeniae* emit CH_4 *in vivo* (Depkat-Jakob *et al.* 2012) but functional links to other anaerobes such as fermenters or acetogens that provide H_2, CO_2, or acetate for methanogenesis are unknown.

Similar processes occur in diverse anoxic habitats and those processes seem to be linked to different taxa, indicating a functional redundancy of microorganisms. The production of CH_4 has been well studied in diverse anoxic habitats but intermediary trophic links and microorganisms involved in the methanogenic food web are less studied and mostly conceptualized (Karsten and Drake 1995, Kotsyurbenko *et al.* 1996, Glissmann and Conrad 2000, Bräuer *et al.* 2004, Drake *et al.* 2009, Wüst *et al.* 2009a, Schmidt *et al.* 2015). Mire 'soil', the rhizosphere of mire plants, and the gut of the earthworm *E. eugeniae* are examples of such CH_4-emitting anoxic habitats. The following hypothesis was formulated based on published literature:

Methanogenic food webs of contrasting CH_4-emitting habitats are driven by functionally redundant anaerobes.

The objectives of this dissertation were to (a) resolve the complex methanogenic food webs of mire soils, mire rhizosphere, and gut contents from the earthworm *E. eugeniae* and (b) determine if those contrasting methanogenic food webs are driven by functional redundant anaerobes. The bacterial and the methanogenic communities were analyzed by cultivation-dependent, analytical and molecular approaches, including isolation, supplementation of anoxic slurries, determination of dissimilation products, quantification of cultivable microorganisms, stable isotope probing, quantification of gene copy numbers, analysis of 16S rRNA and 16S rRNA genes, and analysis of structural genes.

4 Material and Methods

4.1 Sampling sites

4.1.1 Mires 1 and 3, Oberpfalz

Mires 1 and 3 were located 410 m above sea level near Grafenwöhr in the Oberpfalz (Germany).

Figure 7: Images of mire 1 (A) and mire 3 (B).
Images were taken by S. Hunger.

The vegetation of mire 1 consists of wood sorrel (*Oxalis acetosella*), remote sedge (*Carex remota*), stinging nettle (*Urtica dioica*), hairy chervil (*Chaerophyllum hirsutum*), cluster dock (*Rumex conglomeratus*), spruce (*Picea abies*), and true forget-me-not (*Myosotis palustris*). Mire 3 is dominated by peat moss (*Sphagnum* sp.) with a few rushes (*Juncus conglomeratus*), and is surrounded by moor grass (*Molinia caerulea*), birch (*Betula* sp.), heather (*Calluna vulgaris*), and pine (*Pinus* sp.).

4.1.2 Mire 2, Fichtelgebirge

Mire 2 is located 700 m above sea level in the Lehstenbach catchment of the Fichtelgebirge (Germany). The vegetation is dominated by moor grass (*M. caerulea*), sedges (*Carex rostrata*, *Carex nigra*, *Carex canescens*), rushes (*Juncus effusus*), sheathed cotton

sedge (*Eriophorum vaginatum*), and peat moos (*Sphagnum* sp.) (Gerstberger 2001, Paul *et al.* 2006).

Figure 8: Images of mire 2 (A) and a vertical cut of the mire 'soil' (B).
Images were taken by S. Hunger.

The mire is surrounded by spruce (*Picea abies*) (Gerstberger 2001). Samples included whole soil cores, roots of specific plants and root-free soils from the same patches where plant roots were taken. Whole soil cores contained soil and plant roots, and such samples were termed 'soil' hereafter.

4.1.3 Mire 4, Erzgebirge

Mire 4 is located 936 m above sea level in the Wilzsch and the Rolava catchment of the Erzgebirge (Germany). The vegetation consists of sheathed and common cotton sedge (*Eriophorum vaginatum*, *Eriophorum angustifolium*), sedges (*Carex pauciflora*, *Carex limosa*), black crowberry (*Empetrum nigrum*), bog bilberry (*Vaccinium uliginosum*, *Vaccinium oxycoccos*), peat moos (*Sphagnum cuspidatum*, *Sphagnum fuscum*, *Sphagnum balticum*, *Sphagnum dusenii*), bog rosemary (*Andromeda polifolia*), and mountain pine (*Pinus mugo*) (Rentsch and Zitzewitz 2005).

Figure 9: Image of mire 4.

Image was taken by S. Hunger.

4.1.4 Forest, Koke'e State Park

Koke'e State Park is located northwest on Kaua'i (Hawaii, USA). Soil was taken from a forest. Soil of this area developed from volcanic rock (for further site description see Küsel *et al.* [2002]).

4.2 The earthworm *Eudrilus eugeniae*

Specimens of the earthworm *Eudrilus eugeniae* (*Eudrilidae*) were obtained from the distributer Minhobox (Juiz de Fora, Minas Gerais, Brazil) in September 2011 and May 2012.

Figure 10: Image of the earthworm *E. eugeniae*.

Image was taken by Dr. P. S. Depkat-Jakob.

4.3 Sampling procedure

Mires were sampled with a soil corer or a spade. Samples were taken from a depth of 5-30 cm, included soil and roots, and were termed 'soil' samples. Samples of specific plant roots were taken from mire 2. Plants were identified on site and their roots collected. Roots were cut once to separate them from stem and leaves, and were collected together with soil that was attached to the roots. Some *Carex* samples could not be assigned to a specific species because collection took place when *Carex* was not in bloom, and such samples were termed *Carex* sp. which were *C. nigra*, *C. rostrata*, or *Carex canescens*. Samples for enrichment of isolates were obtained from patches with equal distribution of *Carex* sp. and *M. caerulea*. Samples were stored in airtight sterile plastic bags on ice for slurry experiments and chemical analysis or stored in liquid dinitrogen (N_2) for molecular analysis. Samples were collected on the same day at which incubations were started.

Earthworms were kept on composted cow manure for at least 60 hours in the dark before use. Adult earthworms that were used for the analysis were approximately 2.3 ± 0.2 g and 11-20 cm in length (Schulz *et al.* 2015). Gut contents was squeezed out of washed earthworms under O_2-minimized conditions (Depkat-Jakob *et al.* 2012, Schulz *et al.* 2015).

4.4 Solutions, media, and buffer

Solutions, media, and buffer were prepared with deionized water (Seralpur Pro CN, Seral Erich Alhäuser, Ransbach-Baumbach, Germany). For preparation of anoxic solutions, deionized water was boiled for approximately 30 minutes and was cooled down during flushing with 100 % CO_2 or 100 % N_2 (Hungate 1969, Daniel and Drake 1993). Anoxic solutions were stored in gastight serum bottles that were sealed with rubber stoppers and crimps. All anoxic and oxic solutions were sterilized by autoclaving (1 bar overpressure, 120 °C, 25 minutes; autoclave, Adolf Wolf SANOclav, Bad Überkingen, Germany) or filter sterilization (0.2 µm pore size). The pH of oxic and anoxic solutions was adjusted with oxic or anoxic HCl, NaOH or bicarbonate solutions (4.8.3).

4.4.1 Defined mineral medium DM1 (anoxic)

Anoxic defined mineral medium DM1 was prepared according to Hunger *et al.* (2015).

Mineral salts in mg L^{-1}:

KH_2PO_4	10
NH_4Cl	4.6
$MgCl_2$•6 H_2O	10
$CaCl_2$•2 H_2O	10

Trace metals in mg L^{-1}:

$MnSO_4$•H_2O	2.5
$FeCl_2$•4 H_2O	0.7
$CoCl_2$•2 H_2O	1
$CaCl_2$•2 H_2O	1
$ZnCl_2$	0.5
$AlK(SO_4)_4$•12 H_2O	0.2
H_3BO_3	0.1
Na_2MoO_4•2 H_2O	0.1
$CuSO_4$•5 H_2O	0.1
Na_2WO_4•2 H_2O	0.05
$NiCl_2$•2 H_2O	0.2
H_2SeO_3	0.5

Vitamins in mg L^{-1}:

pyridoxal HCl	0.05
thiamine HCl	0.25
riboflavin	0.25
nicotinic acid	0.25
calcium D-pantothenate	0.25
p-aminobenzoic acid	0.25

lipoic acid	0.15
biotin	0.1
folic acid	0.1
cyanocobalamin	0.25

The pH was adjusted according to the *in situ* pH of the corresponding mire pore water.

4.4.2 Defined mineral medium DM2 (anoxic)

Anoxic defined mineral medium DM2 was prepared modified from Balch *et al.* (1979), Paul *et al.* (2006), and Wüst *et al.* (2009a).

Mineral salts in mg L^{-1}:

$(NH_4)_2SO_4$	12.6
Na_2SO_4	13.5
$CaCl_2$•2 H_2O	10
$MgCl_2$•6 H_2O	10
$FeCl_2$•4 H_2O	10
KH_2PO_4	0.4

Trace metals in mg L^{-1}:

$C_6H_6NNa_3O_6$• H_2O	15
$MnSO_4$• H_2O	5
$FeSO_4$•7 H_2O	1
$CoCl_2$•6 H_2O	1
$CaCl_2$•2 H_2O	1
$ZnSO_4$•7 H_2O	1
$AlK(SO_4)_2$•12 H_2O	0.2
$CuSO_4$•5 H_2O	0.1
H_3BO_3	0.1
Na_2MoO_4•2 H_2O	0.1

Concentration of vitamins were as described with defined mineral medium DM1 (4.4.1). The pH was adjusted to 4.8.

4.4.3 Reduced undefined medium RU1 (anoxic)

Anoxic reduced undefined medium RU1 was prepared according to Hunger *et al.* (2016). Concentration of vitamins were as described with defined mineral medium DM1 (4.4.1).

Mineral salts in mg L^{-1}:

KH_2PO_4	500
NH_4Cl	400
NaCl	400
$MgCl_2$•6 H_2O	50
$CaCl_2$•2 H_2O	10

Trace metals in mg L^{-1}:

$C_6H_6NNa_3O_6$•H_2O	7.5
$MnSO_4$•H_2O	2.5
$FeSO_4$•7 H_2O	0.5
$Co(NO_3)_2$•6 H_2O	0.5
$ZnCl_2$	0.5
$AlK(SO_4)_4$•12 H_2O	0.05
H_3BO_3	0.05
Na_2MoO_4•2 H_2O	0.05
$CuSO_4$•5 H_2O	0.05
Na_2WO_4•2 H_2O	0.05
$NiCl_2$•2H_2O	0.25
H_2SeO_3	0.25

Supplements in mg L^{-1}:

cysteine	250
Na_2S•7-9 H_2O	250
resazurin	1
$NaHCO_3$	7,500
yeast extract	500 or 1,000

The pH was adjusted to 5.0 or 6.8.

4.4.4 Reduced undefined medium RU2 (anoxic)

Anoxic reduced undefined medium RU2 was prepared according to Hunger *et al.* (2016).

Supplements in mg L^{-1}:

cysteine	250
Na_2S•7-9 H_2O	250
resazurin	1
$NaHCO_3$	7,500
yeast extract	500 or 1,000

Mineral salts, trace metals, and vitamins were as described with defined mineral medium DM1 (4.4.1). Additionally, 50 mL root extract L^{-1} (4.4.11) were added. The pH was adjusted to 5.0 or 6.8. Supplemented root extract was prepared from roots of mire 2.

4.4.5 Reduced undefined medium RU3 (anoxic)

Anoxic reduced undefined medium RU3 was prepared modified from Balch *et al.* (1979), Daniel *et al.* (1990), and Wüst *et al.* (2009a). Mineral salts, trace metals, and vitamins were as described with defined mineral medium DM1 (4.4.1).

Supplements in mg L^{-1}:

cysteine	250
Na_2S•7-9 H_2O	250
resazurin	1
$NaHCO_3$	7,500
yeast extract	500 or 1,000

The pH was adjusted to 6.8. Additionally, 10 mL worm extract L^{-1} (4.4.13) were added after autoclaving.

4.4.6 Reduced undefined medium RU4 (anoxic)

Anoxic reduced undefined medium RU4 was prepared modified from Balch *et al.* (1979) and Wüst *et al.* (2009a). Mineral salts, trace metals, and vitamins were as described with defined mineral medium DM1 (4.4.1).

Supplements in mg L^{-1}:

cysteine	250
Na_2S•7-9 H_2O	250
resazurin	1
$NaHCO_3$	7,500
yeast extract	500
tryptone	500

The pH was adjusted to pH 6.8. Additionally, 10 mL worm extract L^{-1} (4.4.13) were added after autoclaving.

4.4.7 Undefined mineral medium UM1 (oxic)

Oxic undefined mineral medium UM1 was prepared according to Hunger *et al.* (2016). Mineral salts and trace metals were as described with reduced undefined medium RU1 (4.4.3). Vitamins were as described with defined mineral medium DM1 (4.4.1).

Supplements in g L^{-1}:

yeast extract	0.5 or 1

The pH was adjusted to 5.0.

4.4.8 Undefined mineral medium UM2 (anoxic)

Anoxic undefined mineral medium UM2 was prepared according to Hunger *at al.* (2015). Mineral salts, trace metals, and vitamins were as described with defined mineral medium DM1 (4.4.1).

Supplements in g L^{-1}:

yeast extract	0.5
tryptone	0.5

Additionally, 50 mL root extract L^{-1} (4.4.11) or 50 mL soil extract L^{-1} (4.4.12) were added. Supplemented extracts were prepared from ‘soil’ of mire 1 for ‘soil’ slurries from mire 1, from roots of mire 2 for ‘soil’ slurries from mire 2, from roots of mire 3 for ‘soil’ slurries from mire 3, and from roots of mire 4 for ‘soil’ slurries from mire 4. The pH was adjusted according to the *in situ* pH of the corresponding mire pore water.

4.4.9 Undefined mineral medium UM3 (oxic)

Oxic undefined mineral medium UM3 was prepared according to Hunger *et al.* (2015). Mineral salts, trace metals, and vitamins were as described with defined mineral medium DM1 (4.4.1).

Supplements in g L^{-1}:

yeast extract	0.5
tryptone	0.5

Additionally, 50 mL root extract L^{-1} (4.4.11) or 50 mL soil extract L^{-1} (4.4.12) were added. Supplemented extracts were prepared from ‘soil’ of mire 1 for ‘soil’ slurries from mire 1, from roots of mire 2 for ‘soil’ slurries from mire 2, from roots of mire 3 for ‘soil’ slurries from

mire 3, and from roots of mire 4 for 'soil' slurries from mire 4. The pH was adjusted according to the *in situ* pH of the corresponding mire pore water. After autoclaving, 0.5 g cycloheximide L^{-1} were added to inhibit growth of eukaryotes.

4.4.10 Undefined mineral medium UM4 (anoxic)

Anoxic undefined mineral medium UM4 was prepared according to Balch *et al.* (1979) and Daniel *et al.* (1990).

Trace metals in mg L^{-1}:

$C_6H_6NNa_3O_6 \cdot H_2O$	7.5
$MnSO_4 \cdot H_2O$	2.5
$FeSO_4 \cdot 7\ H_2O$	0.5
$Co(NO_3)_2 \cdot 6\ H_2O$	0.5
$ZnCl_2$	0.5
$NiCl_2 \cdot 6\ H_2O$	0.25
$CuSO_4 \cdot 5\ H_2O$	0.05
$AlK(SO_4)_2 \cdot 12\ H_2O$	0.05
H_3BO_3	0.05
$Na_2MoO_4 \cdot 2\ H_2O$	0.05

Supplements in mg L^{-1}:

resazurin	1
$NaHCO_3$	3,500
yeast extract	1,000

The pH was adjusted to 6.8.

4.4.11 Root extract (oxic)

A mixture of roots was collected from mire 'soil' cores (mires 2-4, 4.1.1, 4.1.2, 4.1.3) and one root extracts per mire was prepared according to Hunger *et al.* (2015). Roots were washed extensively to remove soil particles. 'Soil' cores of mire 1 contained very little roots

and no root extract was prepared. Root extracts were prepared by grinding root of approximately 300 g fresh weight with a mortar and pestle in liquid nitrogen. Deionized water was added to ground roots to a final volume of one liter. Solutions with ground roots were autoclaved, incubated for one week and filtered to remove particles resulting in a clear root extract. Root extracts were utilized as supplements to medium that was used to prepare slurries (4.4.1) and serial dilutions (4.6.1, 4.7).

4.4.12 Soil extract (oxic)

Mire 1 contained very little roots, thus a soil extract was prepared according to Hunger *et al.* (2015). The soil extract was prepared by mixing 500 mL deionized water with 500 g soil. The solution with soil was autoclaved, incubated for one week and filtered to remove particles resulting in a clear soil extract.

4.4.13 Worm extract (anoxic)

E. eugeniae was not accessible in Germany, therefore the earthworm *Lumbricus terrestris* was used to obtain a worm extract. Approximately 60 g earthworms were washed with sterile water and numbed on ice before shredding in a blender for 60 seconds twice. The blender was cooled on ice before and in between blending. Shredded worms were diluted with 120 mL sterile deionized water and incubated on an end-over-end shaker at 4 °C for approximately 12 hours. Worm extract was centrifuged for 10 minutes by 5,000 *g*. Supernatant was sterile filtrated into a sterile serum bottle, gas-tight sealed with sterile butyl rubber stoppers and flushed with sterile 100 % argon for 30 minutes.

4.4.14 Solidified reduced undefined medium RU1 (anoxic)

Solidified reduced undefined medium was prepared modified from reduced undefined medium RU1 (4.4.3, Hunger *et al.* 2016) with the addition of 10 g gelrite L^{-1} and 50 mL root extract L^{-1}. Supplemented root extract was prepared from roots of mire 2. The pH was adjusted to 5.0.

4.4.15 Solidified undefined mineral medium UM4 (anoxic)

Anoxic solidified undefined mineral medium UM4 was prepared according to undefined mineral medium UM4 (4.4.10, modified from Balch *et al.* [1979] and Daniel *et al.* [1990] with the addition of 15 g agar L^{-1}. The pH was adjusted to 6.8.

4.4.16 Semi-solid medium RU1 (anoxic)

Anoxic semi-solid medium RU1 was prepared modified from reduced undefined medium RU1 (4.4.3, modified from Hunger *et al.* [2016]) with the addition of 2.2 g agarose L^{-1}. The pH was adjusted to pH 6.8.

4.4.17 Semi-solid medium RU3 (anoxic)

The semi-solid medium was prepared according to the reduced undefined medium RU3 (4.4.5, modified from Balch *et al.* [1979], Daniel *et al.* [1990], and Wüst *et al.* [2009a]) with the addition of 2.2 g agarose L^{-1}. The pH was adjusted to 6.8.

4.4.18 LB agar plates with ampicillin

LB agar plates with ampicillin were prepared modified from Sambrook *et al.* (1989).

In g L^{-1}:

tryptone	10
yeast extract	5
NaCl	5
agar	15

Additionally, sterile filtrated solutions of ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were added to warm solution after autoclaving and before solidifying, resulting in a final concentration of (in mg L^{-1}):

ampicillin	100
IPTG	120
X-gal	40

The pH was adjusted to 7.0. LB agar plates that were used with pJET1.2/blunt vector plasmid-containing *E. coli* lacking IPTG or X-gal.

4.4.19 SOC medium

SOC medium was prepared according to Sambrook *et al.* (1989).

In mg L^{-1}:

tryptone	2,000
yeast extract	500
NaCl	60
KCl	20

Additionally, sterile filtrated solutions of $MgCl_2$, $MgSO_4$, and glucose were added to medium after autoclaving, resulting in a final concentration of (in mg L^{-1}):

$MgCl_2$	200
$MgSO_4$	250
glucose	360

The pH was adjusted to 7.0.

4.4.20 Diethylpyrocarbonate-treated deionized water

Deionized water was treated with 10 mL diethylpyrocarbonate (DEPC) L^{-1} at 37 °C for 4 hours to inactivate RNases (Sambrook *et al.* 1989). DEPC was inactivated by autoclaving.

4.4.21 PCR-water

Deionized water was sterile filtrated to remove particles and autoclaved to inactivate DNases.

4.4.22 Crystal violet

Crystal violet was prepared modified from Bast (1999), by dissolving 15 g of crystal violet in 100 mL 96 % ethanol at 50 °C. The cold solution was filtrated (0.2 µm pore size). On a per liter basis, 250 mL crystal violet-ethanol solution and 10 g ammonium oxalate monohydrate were mixed.

4.4.23 Safranin

Safranin was prepared according to Bast (1999), by dissolving five grams of safranin in 100 mL 96 % ethanol at 50 °C. The cold solution was filtrated (0.2 µm pore size) and diluted 1:10 with deionized water.

4.5 Anoxic incubations of environmental samples

4.5.1 Preparation of anoxic slurries

Slurries were prepared from the following environmental samples: whole soil cores (i.e., 'soil'), root-free soils, soil-free roots of *Carex* sp. and *M. caerulea*, and gut contents of the earthworm *E. eugeniae*.

'Soil' from one sampling point was manually homogenized and used directly for experiments. Plant roots and attached soils were handled in an O_2-free chamber (Mecaplex, Grenchen, Switzerland) until transferred to serum bottles. Soil was separated from roots with a sieve and did not contain any detectable roots; this soil was utilized as root-free soil. Roots were washed extensively with sterile anoxic water until all soil particles were removed. Earthworms were washed and exposed to ice-cold, sparkling mineral water. Gut contents (approximately 25 g) was squeezed out from approximately 100 earthworms while gassing with 100 % argon to minimize exposure of the gut contents to air. Gut contents was homogenized and kept under an anoxic atmosphere of 100 % argon.

Environmental samples were added to infusion flasks, were sealed with rubber stoppers and crimps, and flushed with sterile gas (100 % helium or N_2 depending on the

detector used for gas analysis). If not otherwise mentioned, environmental samples were diluted with anoxic solutions or media.

4.5.2 Analysis of functional redundancy in contrasting mire 'soils'

Most microbiological studies that have investigated the production of CH_4 in mire 'soils' have focused on either a single intermediary process linked to CH_4 production or a single wetland 'soil' (e.g., Kotsyurbenko *et al.* 1996, Bräuer *et al.* 2004, Cadillo-Quiroz *et al.* 2006, Drake *et al.* 2009, Lin *et al.* 2014a, Lin *et al.* 2014b), and little is known about the potential differences and similarities of microbially mediated anaerobic processes and associated microbial communities that drive methanogenesis in contrasting CH_4-emitting mire 'soils'.

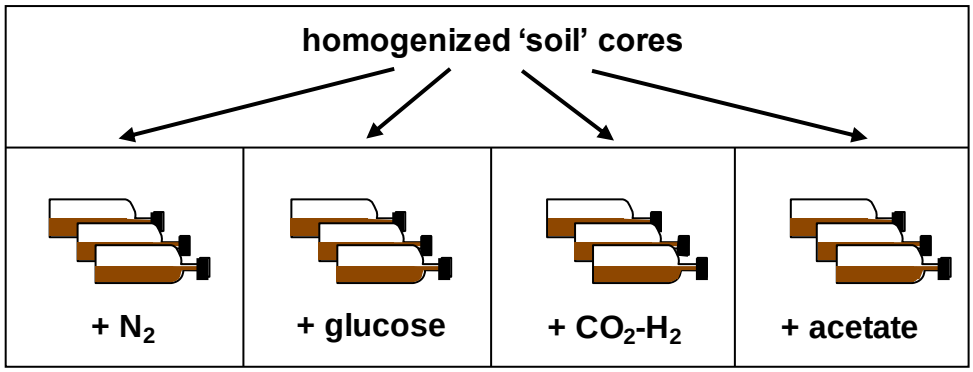

Figure 11: Experimental set-up of anoxic slurries with 'soils' from contrasting mires.

Fresh weight 'soils' from mire 1-4 were diluted with anoxic defined mineral medium DM1 (4.4.1). Treatments were prepared for triplicate analysis. Unsupplemented slurries served as controls. Slurries were incubated in the dark at 15 °C.

Samples were taken in summer or early autumn between June 2011 and August 2013 from mires 1 to 4 (4.1.1, 4.1.2, 4.1.3). Samples were taken from whole soil cores (i.e., 'soil'). 'Soil' samples that were used for the analysis of cultivable microorganisms (4.7) and gene copy numbers (4.10.10) were collected within nine days from all mires. Three 'soil' cores per mire were analyzed that were 4-100 m apart from each other.

For preparation of anoxic slurries, 10 g of fresh weight 'soil' were placed in sterile 250 mL-infusion flasks and were diluted with 35 mL anoxic defined mineral medium DM1 (4.4.1). The pH of DM1 was adjusted according to the pH of the corresponding mire pore water (Table 24). Slurries were either treated with 5 mM glucose, 0.3 mM acetate or 10 mL of

100 % H_2 and CO_2 gas (4:1)(Figure 11). Glucose, H_2 and CO_2 were supplemented once at the beginning of incubation, acetate was supplemented repeatedly every week. Unsupplemented slurries served as controls. Treatments and controls were prepared in triplicates. Slurries were incubated at 15 °C in the dark. Samples for molecular analysis of bacterial 16S rRNA genes and *mcrA* (encodes alpha-subunit of methyl-CoM reductase of methanogens) were collected at the beginning of the incubation and after 21 days of supplementation.

4.5.3 Analysis of FHL activity, methanogenesis and acetogenesis with roots of mire-derived plants

Formate is one of the most important organic acids that is released from the roots of wetland plants (Koelbener *et al.* 2010) and it is a substrate that can be rapidly utilized by mire methanogens, acetogens, and converted to H_2 and CO_2 by FHL-containing taxa (Hunger *et al.* 2011a). Methanogens are associated with rice roots (Conrad and Klose 1999, Chin *et al.* 2004), and the availability of root-derived methanogenic substrates such as formate suggests that methanogens might also be associated with other wetland plant roots.

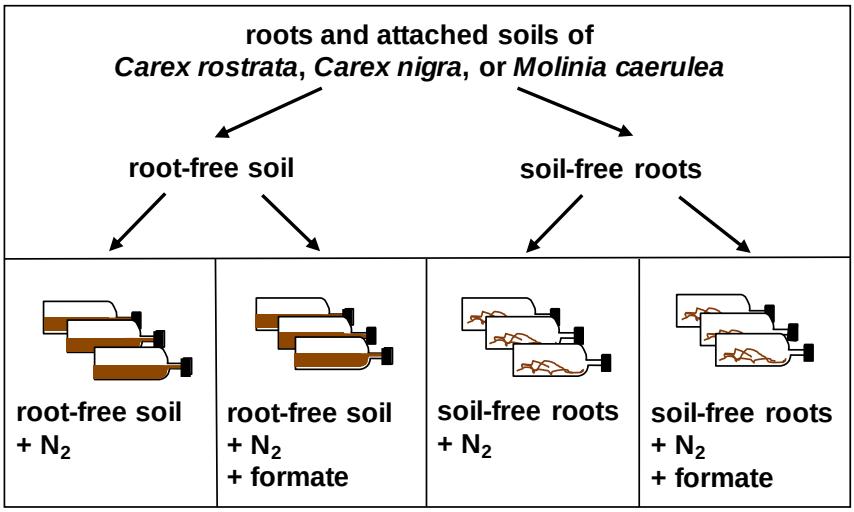

Figure 12: Experimental set-up of formate-supplemented root-free soil slurries and soil-free root slurries from *C. rostrata*, *C. nigra* and *M. caerulea*.

Roots and soils of *C. rostrata*, *C. nigra*, and *M. caerulea* were collected from mire 2 and were separated in an anoxic chamber. Fresh weight soils or roots were diluted with anoxic defined mineral medium DM1 (4.4.1). Slurries were treated with formate. Unsupplemented slurries served as controls. Replication of experiments can be found in legend of corresponding figures in the Results section. Slurries were incubated in the dark on an end-over-end shaker at 15 °C.

Samples of plant roots and attached soils from *C. rostrata*, *C. nigra*, *M. caerulea* or a mixture of roots were taken in October 2010, April and October 2012, and April 2013 from mire 2 (4.1.2). Samples were taken 1-20 m apart from each other. Replicates in treatments reflect replicates of plant patches. Two grams of fresh weight soil-free roots or root-free soils were added to 120-mL infusion flasks in an anoxic chamber. Unless otherwise stated, roots and soils were diluted 1:10 (w/v) with anoxic defined mineral medium DM1 (pH 4.5, 4.4.1). Slurries with soil-free roots or root-free soils were supplemented with 1-5 mM formate for triplicate or sextuplicate analysis (details can be found in corresponding figure legends). Unsupplemented slurries served as controls (Figure 12). Slurries were incubated at 15 °C in the dark on an end-over-end shaker to ensure that roots were covert with medium. Samples for molecular analysis of *mcrA* were collected at the beginning of the incubation with *C. rostrate* roots and after 28 days of supplementation.

4.5.4 'Stable isotope probing' of mire 'soil' with [^{13}C]formate

Supplemental formate stimulated the production of acetate and CH_4 in anoxic slurries with 'soil' from mire 2 (Wüst *et al.* 2009a), which was indicative for acetogenesis and methanogenesis in this mire 'soil', respectively. Different methanogens were detected by molecular analysis but acetogens remained unknown (Wüst *et al.* 2009a). A DNA stable isotope probing (SIP) experiment with anoxic [^{13}C]formate-supplemented 'soil' slurries (5.3) was conducted to identify active acetogens and methanogens. The identification of active methanogens based on the analysis of *mcrA* and active bacterial taxa based on 16S gene analysis was part of my diploma thesis (Hunger *et al.* 2011a). The analysis of active acetogens by obtaining bacterial *fhs* sequences (encodes the formyltetrahydrofolate synthetase, 4.10.9, 4.11.1) from aforementioned 'soil' slurries and the determination of the content of ^{13}C-enriched acetate (4.8.9) was part of my doctoral dissertation.

Three whole soil cores (i.e., 'soil') were taken from a depth of 0-20 cm in July 2008 (4-5 m apart) from mire 2 (4.1.2). The 'soil' was homogenized. Thirty-five grams of fresh weight homogenized 'soil' were placed in sterile 500-Ml infusion flasks and diluted with 125 mL

anoxic defined mineral medium DM2 (pH 4.8, 4.4.2). Slurries were preincubated to remove alternative electron acceptors such as nitrate, sulfate and iron(III), and thus minimize labeling of microorganisms that could anaerobically respire formate (Figure 13).

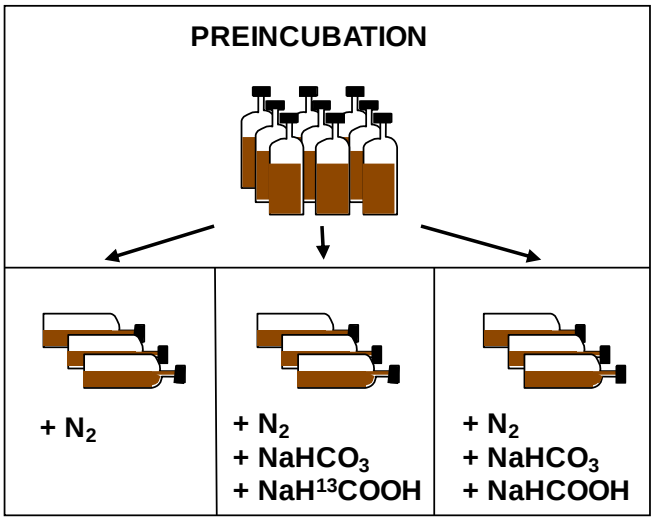

Figure 13: Experimental set-up of formate-supplemented 'soil' slurries.

Fresh weight 'soil' from mire 2 was diluted with anoxic defined mineral medium DM2 (4.4.2). Anoxic slurries were preincubated for 15 days to reduce alternative electron acceptors. After preincubation, slurries were treated with [^{13}C]formate or [^{12}C]formate and additionally with $^{12}CO_2$ in triplicates. Unsupplemented slurries served as controls. Slurries were incubated in the dark at 15 °C.

After 15 days of preincubation, sodium[^{13}C]formate (99 at % ^{13}C) and sodium[^{12}C]formate-supplemented slurries were pulsed daily with approximately 64 µmol formate per slurry. Control slurries lacked supplemental formate. [^{13}C]formate could be converted to $^{13}CO_2$ and H_2, resulting in potentially labelled $^{13}CO_2$ in the gas phase. Two safeguards against CO_2 cross-feeding were taken: (a) formate treatments were pulsed daily with 192 µmol $^{12}CO_2$ (i.e., sodium [^{12}C]bicarbonate) per slurry, and (b) the gas phases of slurries were exchanged with sterile 100 % N_2 before substrate pulsing was initiated and every subsequent fourth day. For exchanging the gas phase with N_2, slurries were evacuated under sterile conditions for 30 minutes at approximately -800 mbar, followed by replacement of the gas phase with 100 % N_2. This procedure was repeated after 15 minutes. Finally, slurries were flushed with sterile 100 % N_2 for 20 minutes. The pH was adjusted every fourth day to approximately pH 4.5 with anoxic sterile five molar HCl. Slurries were incubated for 39 days

horizontally in the dark at 15 °C. Samples for DNA SIP were taken after preincubation (i.e., day 16) and after 24 days of formate-supplementation (i.e., day 39) (4.10.7).

4.5.5 'Stable isotope probing' of gut contents derived from the earthworm *E. eugeniae* with [^{13}C]glucose

The earthworm gut is an anoxic and saccharide-rich habitat that harbors denitrifiers and fermenters (Drake and Horn 2007, Karsten and Drake 1997, Wüst *et al.* 2009b), suggesting that guts of other earthworms have similar capacities. The earthworm *E. eugeniae* not only emits N_2O and H_2 that are indicative for denitrification and fermentation but also emits CH_4 that is indicative for methanogenesis (Depkat-Jakob *et al.* 2012, and unpublished data). A methanogenic enrichment derived from gut contents of *E. eugeniae* (4.6.3) displayed the capacity for methanogenesis along with the capacity for acetogenesis (Figure 50). Based on this observation a RNA stable isotope probing experiment with [^{13}C]glucose- and H_2-supplemented gut contents of *E. eugeniae* was conducted to identify active acetogens.

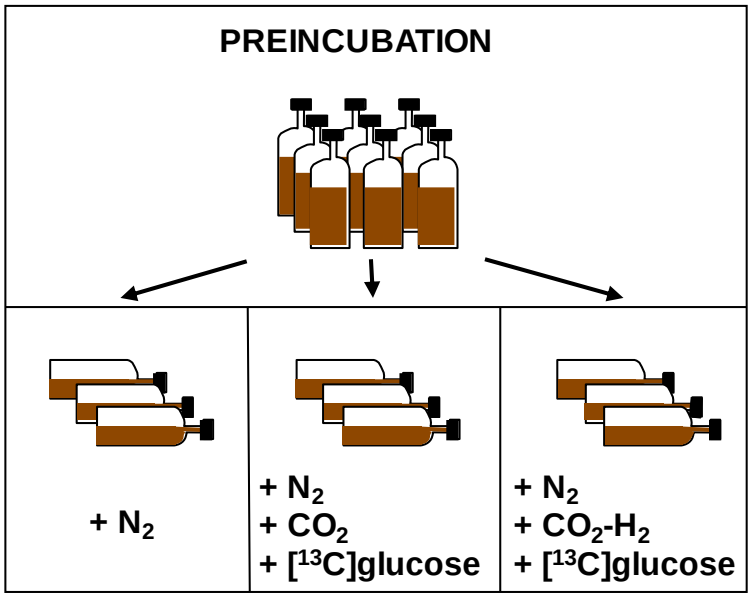

Figure 14: Experimental set-up of glucose-supplemented slurries with gut contents of *E. eugeniae*.

Anoxic gut contents of *E. eugeniae* was preincubated for three days to remove alternative electron acceptors. After preincubation, fresh weight gut contents was diluted with anoxic sodium phosphate buffer. Slurries were treated with CO_2 and [^{13}C]glucose, or CO_2, [^{13}C]glucose and H_2 for triplicate analysis. Unsupplemented slurries served as controls. Slurries were incubated in the dark at 25 °C.

Gut content of *E. eugeniae* was collected (4.5.1) and preincubated under a 100 % argon atmosphere in a sterile, gas-tight serum bottle at room temperature for three days to reduce alternative electron acceptors (Figure 14). On the fourth day, sterile 120-mL serum bottles were filled with one gram of preincubated gut contents in an anoxic chamber (100 % N_2 atmosphere). Gut contents in serum bottles was diluted with nine milliliters of sterile, anoxic sodium phosphate buffer (15 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 7.0). Six slurries were pulsed twice a day with 350 µM [^{13}C]glucose (Sigma-Aldrich Chemie GmbH, 99 at % ^{13}C) and had approximately five percent $^{12}CO_2$ added to the gas phase once at the beginning of the incubation. Approximately 10 % H_2 were added once at the beginning of the incubation to three of the [^{13}C]glucose-supplemented slurries to additionally stimulate acetogenesis. Three unsupplemented slurries served as controls. Slurries were incubated at 25 °C in the dark. Samples for RNA SIP were taken after preincubation (i.e., day 4) and after seven days of glucose-supplementation (i.e., day 10) (4.10.7).

4.6 Enrichment and isolation procedures

4.6.1 Enrichment and isolation of FHL-containing bacteria, fermenters, and an acetogen from mire-derived roots

Carex and *Molinia* roots transformed formate to H_2 and CO_2, and likewise displayed the potential for hydrogenotrophic acetogenesis (Figure 33). A mixture of soil-free *Carex* and *Molinia* roots from mire 2 (4.1.2) were used to enrich and isolate bacteria potentially associated with these activities. The enrichment medium contained H_2 and formate in order to maximize the likelihood of obtaining such microorganisms.

Roots were incubated in reduced undefined medium RU2 (without root extract, 4.4.4) in the dark at 15 °C. The gas phase was 100 % CO_2. The medium of early enrichments contained 10 mM 2-bromoethanesulfonic acid to inhibit methanogenesis, 5 mM formate, and approximately 10 % H_2 at pH 5. Later incubations were performed without 2-bromoethanesulfonic acid at pH 6.8 to increase growth. At the beginning, whole roots were transferred and served as inoculum. After three transfers of roots, the medium was

supplemented with five percent root extract and the liquid phase served as inoculum for further enrichments. The final enrichment was designated FH (because formate and H_2 were provided with the intent to enrich formate- and H_2-utilizing bacteria). The initial root enrichment converted approximately 54 mmol formate L^{-1} and 87 mmol H_2 L^{-1} as co-substrate to 36 mmol acetate L^{-1}.

Isolates SB1 (*Citrobacter*-related) and SB2 (*Hafnia*-related) were obtained by (a) plating 1:10 serial dilutions of enrichment FH on formate-supplemented solidified reduced undefined medium RU1 (H_2-CO_2 gas phase, pH 5.0, 4.4.14), (b) transferring single colonies to liquid reduced undefined medium RU1 (pH 5.0, 4.4.3), and (c) repeating steps a and b two more times. Solidified and liquid reduced undefined medium RU1 contained five millimolar formate. The gas phase of serum bottles with liquid reduced undefined medium RU1 contained approximately 10 % H_2. Characterization of the isolates was performed in oxic undefined mineral medium UM1 (pH 5, 4.4.7) or reduced undefined medium RU1 (pH 5, 4.4.3). Incubations were performed in the dark at 15 °C.

Isolates SB3 (*Clostridium*-related) and isolate SB4 (*Carnobacterium*-related) were obtained by (a) inoculating glucose-supplemented semi-solid medium (pH 6.8, 4.4.16) with 1:10 serial dilutions of the acetogenic enrichment FH, (b) transferring single colonies to liquid medium (pH 6.8, 4.4.3), and (c) repeating steps (a) and (b) four more times. Characterization of the isolates was performed in reduced undefined medium RU1 (pH 5 or pH 6.8, 4.4.3). Incubations were performed in the dark at 15 °C.

Although enrichment FH displayed acetogenic activity, no pure acetogenic isolate was obtained by the aforementioned protocol. Acetogenic activity was lost after a few transfers if the new medium lacked autoclaved roots or sterile root extract (4.4.11). Characterization of the acetogenic enrichment was performed in the reduced undefined medium RU1 at pH 5 and pH 6.8. Incubations were performed in the dark at 15 °C.

4.6.2 Isolation of an acetogen and a fermenter from forest 'soil'

Acetogens are often thought to be obligate anaerobes but have been isolated from habitats that are exposed to O_2 such as the roots of the sea grass *Halodule wrightii* (Küsel *et al.* 2001) or oxic Egyptian soil (Größner *et al.* 1999), indicating that acetogens can be O_2 tolerant to some extent. In this regard, anoxic slurries with forest 'soil' from Koke'e State Park (4.1.4) converted supplemental H_2 and CO_2 to acetate in a stoichiometric ratio that was indicative for acetogenesis (Küsel *et al.* 2002), and thus the objective of this study was to isolate an acetogen from this forest 'soil'.

For initial enrichment, aerated soil from Koke'e State Park was diluted 1:10 (w/v) in undefined mineral medium UM4 (pH 6.8, 4.4.10). This medium was lacking reducing agents to increase the likelihood of obtaining an acetogen with at least a minimal tolerance to O_2. Infusion flasks were incubated horizontally at 30 °C and were not shaken. The acetogenic culture KH (for Kaua'i, Hawaii) was obtained by streaking enrichments on solidified undefined mineral medium UM4 (H_2-CO_2 gas phase, 4.4.15), transferring colonies to liquid undefined mineral medium UM4, and then re-streaking two times. Culture KH formed acetate in response to xylan and raffinose, saccharides that are not normal substrates for known acetogens (Drake *et al.* 2006), and it was suspected that KH might contain more than one microorganism. KHa and KHb were taken from the highest growth-positive dilutions of undefined mineral medium UM4 supplemented with either H_2 or raffinose, respectively. KHa and KHb were then obtained from isolated colonies on solidified undefined mineral medium UM4 (pH 6.7, 4.4.15). Undefined mineral medium UM4 (6.8, 4.4.10) was used for further characterization of KHa and KHb.

4.6.3 Enrichment of methanogens and acetogens from gut contents of *E. eugeniae*

The earthworm *E. eugeniae* emitted CH_4 and displayed the potential of methanogenesis being an active process in the earthworm gut (Depkat-Jakob *et al.* 2012). Attempts were made to isolate methanogens from gut contents of *E. eugeniae* by diluting gut

contents 1:10 (w/v) with reduced undefined medium RU4 (pH 6.8, 4.4.6) in a H_2-CO_2 headspace (approximately 1:9). The enrichment was transferred into new medium RU4 and incubated at 25 °C with a H_2-CO_2 headspace (approximately 1:9). After another transfer of the enrichment to new medium RU4 with a 100 % CO_2 headspace and either 22 mM H_2 or 5 mM acetate supplementation. Enrichment was incubated at 25 °C for 14 days. Unfortunately, further attempts to transfer and enrich the methanogens failed and the enrichment was lost.

The methanogenic enrichment converted H_2 and CO_2 not only to CH_4 but also to acetate, suggesting that the gut contents of *E. eugeniae* harbors acetogens. Thus, gut contents of *E. eugeniae* was diluted 1:10 (w/v) with reduced undefined medium RU3 (pH 6.8, 4.4.5). The gas phase was 100 % CO_2. A serial dilution was prepared. H_2 served as substrate and only highest dilutions that converted H_2 to acetate were selected for further serial dilutions. The enrichment was transferred four times in liquid medium until semi-solid medium was utilized for further enrichment by (a) serial 1:10 dilutions (w/v) of the enrichment prepared with semi-solid medium RU3 (4.4.17), (b) single colonies transferred from semi-solid medium RU3 to liquid reduced undefined medium RU3, and (c) steps (a) and (b) were repeated once more. Characterization of the acetogenic enrichment was performed in reduced undefined medium RU3. Incubations were performed in the dark at 25 °C.

4.7 Determination of cultivable microorganisms

The most probable number (MPN) approach was used to estimate the number of cultivable aerobes and anaerobes in four contrasting mire ‘soils’ (Phelps 1908, Oblinger and Koburger 1975). ‘Soil’ samples were taken 4-100 m apart from each other and were collected within nine days from all mires. Undefined mineral media UM2 (anoxic, 4.4.8) and UM3 (oxic, 4.4.9) were used to estimate the number of microorganisms. Media (4.4.8, 4.4.9) for the 10-fold dilution series did not contain yeast extract or tryptone. The pH of the media UM2 and UM3 was adjusted according to the *in situ* pH of the corresponding mire pore water. Two oxic and two anoxic 10-fold dilution series were prepared from three ‘soil’ samples of each mire. Microbes were dispersed in the first dilution of the 10-fold dilution series with the help of a

dispersion solution (150 g sodium pyrophosphate L^{-1}) and a mechanical procedure (1 hour at 15 °C with 100 cycles min^{-1} on an end-over-end shaker) (Bast 1999). The 10-fold dilution series were used to inoculate wells in quadruplicates in 96-well plates. Preparation of anoxic dilution series was performed in an anoxic chamber (100 % N_2 gas phase). Inoculated 96-well plates were incubated in the dark at 15 °C under anoxic (100 % N_2 gas phase) or oxic conditions for 18 weeks. Wells were scored growth-positive if the optical density increased at least 0.01 units (4.9.4) and growth was visible to the naked eye. Cultivable cell numbers are per gram soil dry weight (i.e., [g $soil_{DW}$]).

4.8 Analytical techniques

4.8.1 Dry weight

Dry weight of environmental samples was determined in triplicates by weighing samples before and after drying at 60 °C for approximately 72 hours.

4.8.2 Sampling and preparation of liquid samples

Gas and liquid phase were sampled with sterile and gas-flushed syringes. Liquid samples were stored at -20 °C for chemical analysis or at -80 °C for molecular analyses. Untreated gut contents of *E. eugeniae* and the substrate that the earthworm was raised on were taken and stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) to stabilize nucleic acids until analyzed.

4.8.3 pH

The pH of liquid samples was determined with a pH electrode (InLab R422, Mettler Toledo GmbH, Gießen, Germany) and a digital pH meter (WTW pH 330, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany).

4.8.4 Quantification of ions by ion chromatography

Nitrate, sulfate, phosphate and chloride were analyzed at the Center for Analytical Chemistry (Bayreuth Center of Ecological and Environmental Research, University of

Bayreuth, Bayreuth, Germany) (Hunger *et al.* 2011a). Nitrate and sulfate were analyzed with a Dx500 ion chromatograph equipped with an ED 40 detector and AS 4A-SC column (Dionex Corporation, Sunnyvale, USA). The mobile phase was 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate at a flow rate of 2 mL min^{-1}. The column temperature was 35 °C.

4.8.5 Quantification of total nitrogen and carbon content

Total nitrogen and total carbon content in solid samples were analyzed at the Center for Analytical Chemistry (Bayreuth Center of Ecological and Environmental Research, University of Bayreuth, Bayreuth, Germany). Solid samples were dried for 48 hours at 60 °C and were ground to powder with a mixer mill (MM200, Retsch, Haan, Germany) before measurement. Total nitrogen and total carbon content of powdered samples were analyzed with an element analyzer (ThermoQuest, Flash EA 1112, Thermo Fisher Scientific, Waltham, USA). Samples were combusted at 900 °C under an O_2 gas flow to CO_2, NO_x, and water. Gases were collected in a helium gas flow with a flow rate of 130 mL min^{-1} and NO_x was reduced in a column with copper oxide to N_2. CO_2 and N_2 were quantified with a thermal conductivity detector (Thermo Quest, Flash EA 1112, Thermo Scientific, Waltham, USA).

4.8.6 Quantification of non-purgeable organic carbon

Non-purgeable organic carbon (NPOC) in liquid samples was analyzed at the Center for Analytical Chemistry (Bayreuth Center of Ecological and Environmental Research, University of Bayreuth, Bayreuth, Germany). Liquid samples were filtrated (0.45 µm pore size) and acidified with two normal HCl before measurement. Acidification of samples dissolved bicarbonates and released them as CO_2. Acidified samples were treated with an inert gas to blow out the CO_2 and other volatile compounds from samples. NPOC was analyzed with a TOC/TNb analyzer (multi N/C 2100, Analytik Jena, Jena, Germany). Samples were combusted at 800 °C under synthetic air gas flow. Produced CO_2 was quantified with a nondispersive infrared sensor NDIR detector (NDIR detector, Analytik Jena, Jena, Germany) which determined the optical dispersion of gases.

4.8.7 Quantification of gases

H_2, CO_2 and CH_4 were measured with gas chromatographs equipped with thermal conductivity detector, helium ionization detector or flame ionization detector (5890 series II, Hewlett-Packard, Palo Alto, USA or SRI Instruments, Torrance, USA) (Küsel and Drake 1995, Hunger *et al.* 2011a). The thermal conductivity detector measured the changes in the thermal conductivity of analytes in the carrier gas in comparison to the thermal conductivity of pure carrier gas. The flame ionization detector measures ions that were formed during ionization of analytes in a H_2 flame. The helium ionization detector measured ions that were formed during ionization of analytes by metastable helium. Concentrations of gases are combined concentrations from gas and liquid phases and were calculated from the ideal gas law (4.12.5) taking into consideration the ambient pressure (Barogeber, 946...1053 hPa, ThiesClima, Göttingen, Germany), overpressure in incubation flasks (DMG 2120, Ballmoos Elektronik AG, Horgen, Switzerland), temperature, pH (4.8.3), and volume of gas and liquid phases in serum bottles, infusion flasks and tubes (Blachnik 1998, Krichevsky and Kasarnovsky 1935). Standard curves were used to calculate the gas concentration in gas samples. Standard curves were prepared by triplicate determination of the area peak derived from known concentrations of seven H_2 standard, seven CH_4 standards, and nine CO_2 standards. In order to compare consumption and production of gases and organic compounds, amounts of gases were given in relation to liquid phases.

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Table 8: Settings for Hewlett Packard 5890 Series II and SRI 8610C gas chromatographs.

	Hewlett Packard 5890 Series II			SRI 8610C		
Gases measured	CO_2	CH_4	H_2, CH_4	CO_2	CH_4, H_2	CH_4
Detector	thermal conductivity detector (TCD)	flame ionization detector (FID)	thermal conductivity detector (TCD)	thermal conductivity detector (TCD)	helium ionization detector (HID)	flame ionization detector (FID)
Column	chromosorb 102, 2 m x 1/8''[a]	molecular sieve, 2 m x 1/8''[a]	molecular sieve, 2 m x 1/8''[a]	HayeSep-D, 2 m x 1/8''[b]	6‘ molecular sieve 13x column[b]	HayeSep-D, 2 m x 1/8''[b]
Carrier gas	helium	helium	argon	helium	helium	helium
Flow rate	15 mL min^{-1}	40 mL min^{-1}	33 mL min^{-1}	25 mL min^{-1}	20 mL min^{-1}	40 mL min^{-1}
Oven temperature	40 °C	60 °C	60 °C	80 °C	80 °C	60 °C
Injector temperature	150 °C	120 °C	150 °C	60 °C	60 °C	60 °C
Detector temperature	175 °C	150 °C	175 °C	175 °C	150 °C	380 °C
Volume injected	0.1-0.2 mL	0.1-0.2 mL	0.1-0.2 mL	1 mL	1 mL	0.4-1 mL
Additional settings	-	-	-	TCD amplifier high	HID current on, 250 °C	FID amplifier high
Lower quantification limit	100 ppm	0.6 ppm	100 ppm (H_2) 1000 ppm (CH_4)	0.5 %	19 ppm (CH_4) 97 ppm (H_2)	19 ppm

[a] Alltech, Unterhaching, Germany.
[b] SRI Instruments, Torrance, USA

4.8.8 Quantification of sugars, ethanol and organic acids

Liquid samples from slurries were centrifuged (5,000 *g*, five minutes) and the supernatant was filtrated (0.2 µm pore size) into a 1.5 mL-glass vial. The glass vial was sealed and used for analysis. Organic acids, sugars and ethanol in liquid samples were determined with a high performance liquid chromatograph (1090 series II, Hewlett Packard, Palo Alto, USA) that was equipped with a variable wavelength UV detector and a refractive index detector (Series 1200, Agilent Technologies, Böblingen, Germany) (Wüst *et al.* 2009b). The UV detector measures the absorption of light from analytes at a wavelength of 210 nm. The refractive index detector measures the refractive index of the analytes in comparison of the refractive index of the pure mobile phase. Twenty microliter sample were injected into the mobile phase (4 mM H_3PO_4, pH 2.5) via an autosampler and the sample-mobile phase mixture was pumped through an ion exclusion column (Rezex ROA Organic Acid H^+ column, 300 x 7.8 mm, Phenomenex, Torrance, USA) with a flow rate of 0.8 mL min^{-1}. The column was heated to 60 °C by an oven. Stronger interactions of an analyte with the column material (sulfonated polystyrol-divinylbenzol-copolymere) leads to a longer retention time of the compound. Standard curves were used to calculate the concentration of compounds in liquid samples. Standard curves were prepared by triplicate determination of the area peak from known concentrations of eight to nine standards per compound.

4.8.9 Determination of [^{13}C]-enriched organic acids and gases by liquid chromatography coupled to isotope ratio mass spectrometry

The ^{13}C content of organic acids was determined by the Max Plank Institute for Terrestrial Microbiology (Prof. R. Conrad, Marburg, Germany) by liquid chromatography coupled to isotope ratio mass spectrometry (Finnigan™ LC IsoLink, Thermo Fisher Scientific, Waltham, USA) (Krummen *et al.* 2004). Organic acids were separated by high performance liquid chromatography, oxidation, and supplementation of acid/catalyst reagents (ammonium peroxodisulfate, phosphoric acid, silver nitrate). Organic compounds were oxidized to CO_2 in an oxidation reactor at 100 °C. CO_2 of the liquid phase was degassed by a helium counter

flow, which was then dried in an on-line gas-drying unit and injected into the mass spectrometer.

4.8.10 Determination of the volume of the liquid and gaseous phases of incubation flasks

Volume of gas and liquid phase are necessary to calculate concentrations of gases and were determined from each incubation flask (i.e., serum bottle, infusion flask and tube). The interface of liquid and gas phase was marked. The volumes were determined under the assumption that one gram of water occupies one mL of volume. The incubation flasks were weighed empty, completely filled with water, and filled with water up to the mark. The volume of the liquid phase was determined by subtracting the weight of the empty incubation flask from the weight of the same incubation flask that was filled up to the mark. The volume of the gas phase was determined by subtracting the weight of the incubation flask that was filled up to the mark from the weight of the same incubation flask that was completely filled. The complete volume of the incubation flask was determined by subtracting the weight of the empty incubation flask from the weight of the same incubation flask that was completely filled.

4.9 Microbiological methods

4.9.1 Gram staining

Gram staining was used to differentiate between microorganisms (Bast 1999). Glass slides were cleaned with 96 % ethanol before 100 µL cell suspension were transferred on top of the slide. Cells were fixated to the glass surface by swiping the slide through a flame three times. Remaining liquid evaporated at room temperature within 30 minutes. Fixated cells were exposed to crystal violet for one minute (4.4.22). Crystal violet penetrates the cell wall and cell membrane. Cells were rinsed and incubated for one minute with Lugol's iodine solution (13 mM iodine, 40 mM potassium iodide). During this step, the chloride anion of crystal violet is exchange with iodine from the Lugol's iodine solution and a water-insoluble violet complex is formed. Cells were rinsed with 96 % ethanol and the violet complex outside of cells and

inside of cells with a thin murein structure are dissolved and washed off. The violet complex inside of cells with a thicker murein structure (stain Gram positive) was maintained and the ethanol dehydrated the murein structure. Cells were washed with deionized water to remove the ethanol. Cells were exposed to safranin for 30-60 seconds (4.4.23) and non-violet cells stained red (stain Gram negative). Cells were rinsed with deionized water and dried at room temperature before microscopy.

4.9.2 Cytochrome *c* oxidase test

Cytochrome *c* oxidase is a part of the electron transport chain of aerobes (Berg *et al.* 2003). Cytochrome *c* oxidase transfers electrons from cytochrome c to O_2 forming water during the process and translocating H^+ across the membrane to form an H^+ gradient (i.e., electrochemical potential) that drives ATPases (Berg *et al.* 2003).

A cell suspension was treated with *N*, *N*, *N*′ ,*N*′-tetramethyl-*p*-phenylenediamine (1 % TMPD, 0.1 % ascorbic acid, 70 Mm phosphate buffer, pH 7.0) for 20-30 seconds (modified from Liu and Jurtshuk 1986). TMPD serves as electron donor for cytochrome c that subsequently reduces cytochrome *c*. Colorless TMPD is oxidized and turns dark-blue, and Oxidase-positive cells dye blue within one minute. Oxidase-negative cells show no or late blue staining.

4.9.3 Catalase test

Most aerobes and facultative aerobes contain catalase to detoxify hydrogen peroxide (Berg *et al.* 2003). Catalase catalyzes the reaction of two molecules hydrogen peroxide to two molecules water and one molecule O_2 (Berg *et al.* 2003). A cell suspension was exposed to 10 % hydrogen peroxide (modified from Madigan and Martinko [2006]). Catalase-positive cells form foam resulting from the formation of O_2. Catalase-negative cells do not form foam.

4.9.4 Optical density

The optical density of cultures in tubes or 96-well plates was measured at a wavelength of 660 nm with spectrophotometers (Milton Roy Spectronic 501, Bausch & Lomb Inc., Rochester, USA or µQuant, BioTek Instruments, Winooski, USA).

4.9.5 Microscopy

Cell morphology and mobility of microorganisms in cultures were observed with a microscope (Axioskop 2, Zeiss, Jena, Germany) equipped with an Axiocam MR monochrome (TV 2/3"C 0.63X 1069-414, Zeiss, Jena, Germany). A 1,000-fold magnification was used with bright field to study microorganisms or with phase contrast 3 to determine the Gram staining.

4.10 Molecular techniques

4.10.1 Extraction of DNA and RNA

Roots were ground with a sterile mortar and pestle in liquid nitrogen before nucleic acid extraction. Samples that were stored in RNAlater RNA stabilization reagent were washed three times with RNase-free phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4; centrifugation at 10,000 *g*, 15 minutes) before nucleic acid extraction (Depkat-Jakob *et al.* 2012). Cells from enrichments and pure cultures were harvested by centrifugation (5,000 *g* for five minutes) of 1-10 mL of culture, and discarding of the supernatant resulting in a pellet of cells. All other samples such as ‘soils’ were utilized without prior treatment.

Nucleic acids were extracted by bead-beating lysis, organic solvent extraction, and precipitation (Griffiths *et al.* 2000). Samples were handled on ice during extraction and centrifugation was performed at 4 °C. For extraction of nucleic acids from environmental samples, 110 mg of 0.1 mm glass beads, 150 mg of 0.5 mm glass beads, 35 mg of 1.0 mm glass beads, and two 3.0 mm glass beads (BioSpec Products, Bartlesville, USA) were used. For extraction of nucleic acids from a cell pellet of cultures and ground roots, 35 mg of 0.1 mm glass beads and 35 mg of 0.5 mm glass beads (BioSpec Products, Bartlesville, USA) were

used. Up to 0.5 g of sample, 0.5 mL extraction buffer (RNase- and DNase-free, 5 % cetrimonium bromide, 2 % NaCl, 120 mM potassium phosphate buffer, pH 8.0, 60 °C), and 0.5 mL phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) were added to samples and glass beads. Cells in samples were lysed by bead-beating twice for 30 seconds and 5.5 m s^{-1} (Fast Prep FT120, Thermo Savant, Holbrook, USA). Samples were cooled on ice in between bead-beating. Solid and liquid phases of sample were separated by centrifugation (14,000 *g*, five minutes). Phenol residues in the liquid phase were removed by a chloroform-isoamyl alcohol (24:1) treatment. Samples were homogenized with 0.5 mL chloroform-isoamyl alcohol, centrifuged (14,000 *g*, five minutes), and the upper liquid phase was used for precipitation. Nucleic acids were precipitated with polyethylene glycol (4.10.2) and dissolved in 55 µL DNase- and RNase-free water (DEPC-treated deionized water, 4.4.20) for RNA analysis or TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) for DNA analysis.

For extraction of nucleic acids for quantitative PCR (4.10.10), nucleic acids were extracted twice by bead-beating lysis and organic solvent extraction. Nucleic acid pellets from both extractions were dissolved as described above and were merged to one sample.

4.10.2 Precipitation of nucleic acids with polyethylene glycol

DNA from fractions after isopycnic centrifugation (4.10.7), nucleic acids after extraction (4.10.1), and RNA after digestion of DNA (4.10.5) were precipitated with twice the volume of polyethylene glycol 6000 (30 % polyethylene glycol, 0.1 M HEPES buffer, pH 7.0). Polyethylene glycol for precipitation of DNA from fractions after isopycnic centrifugation was supplemented with 1.6 M NaCl (Neufeld *et al.* 2007b). For an easier visualization of the precipitated pellet with small amounts of nucleic acids, samples were treated with polyethylene glycol and three microliter glycogen (20 mg mL^{-1}). Nucleic acids were precipitated for two hours at room temperature and were centrifuged (14,000 *g*, 30 minutes, 4 or 15 °C). Supernatant was discarded and the nucleic acid pellet was washed twice with RNase- and DNase-free 70 % ethanol. Nucleic acid pellets were dried to completely remove the ethanol.

4.10.3 Precipitation of nucleic acids with isopropyl alcohol

PCR products were precipitated with 0.7-times the volume of 100 % isopropyl alcohol and 0.1-times the volume of five molar NaCl for approximately 12 hours at -20 °C. After precipitation of nucleic acids, samples were centrifuged (13,000 *g*, 30 minutes, 4 °C). Nucleic acid pellets were washed twice with DNase-free 70 % ethanol. The pellets were dried to completely remove ethanol residues.

4.10.4 Precipitation of nucleic acids with sodium acetate buffer

RNA from fractions after isopycnic centrifugation (4.10.7) was precipitated with 0.65 volumes of sodium acetate buffer (3 M, pH 5.2) and five times the volume of RNase-free 96 % ethanol. For an easier visualization of the precipitated RNA pellet, three microliter glycogen (20 mg mL^{-1}) were added additionally. RNA was precipitated for about 12 hours at -20 °C and was centrifuged (14,000 *g*, 30 minutes, 4 or 15 °C). Supernatant was discarded and the RNA pellet was washed twice with RNase-free 70 % ethanol. RNA pellets were dried to completely remove the ethanol.

4.10.5 Separation of RNA by enzymatic digestion of DNA

DNA was removed from nucleic acid extractions with RNase-free DNase according to the manufacturer's instructions (Promega, Mannheim, Germany). Twenty-six microliters of sample with nucleic acids were digested with one unit DNase I in reaction buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.5) for 45 minutes at 37 °C. The reaction was stopped by precipitation of RNA with isopropyl alcohol (4.10.3).

4.10.6 Quantification of nucleic acids

Higher concentrations of nucleic acids (e.g., in PCR products) were quantified with a UV/Vis spectrophotometer (Nano Drop ND-1000, PEQLAB Biotechnologie, Erlangen, Germany). DNA and RNA absorb UV light at a wavelength of 260 nm resulting in an increase of the optical density which is measured with the spectrophotometer and used to calculate DNA and RNA concentrations. Contaminants in the samples are measured at 230 nm and

280 nm. The 260 to 230 ratio and 260 to 280 ratio are used to determine the purity of nucleic acids. Values between 1.8 and 2.0 indicate pure extracts. Lower values indicate the presence of contaminants such as phenols, humic acids, or proteins.

Lower concentrations of DNA (e.g., DNA from fractions after isopycnic centrifugation) were quantified with Quant-iT dsDNA PicoGreen Assay Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. A DNA calibration curve was prepared from fresh reagents and measured each time. Standard and samples were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) in 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium). PicoGreen reagents were added in a 1:1 ratio. Reagents were homogenized by shaking for one minute. Standards and samples were measured with a fluorimeter (FL x 800 Microplate Reader, BioTek Instruments, Bad Friedrichshall, Germany). The fluorescent dye PicoGreen was excited at a wavelength of 485 nm and fluorescence emission intensity was measured at a wavelength of 528 nm. Standards were measured in triplicates and samples in duplicates.

Lower concentrations of RNA (e.g., RNA from fractions after isopycnic centrifugation) were quantified with Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. An RNA calibration curve was prepared from fresh reagents and measured each time. Standards and samples were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) in 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium). PicoGreen reagents were added in a 1:1 ratio. Reagents were homogenized by shaking for one minute. Standards and samples were measured with a fluorescence reader (FL x 800 Microplate Reader, BioTek Instruments, Bad Friedrichshall, Germany). The fluorescent dye RiboGreen was excited at a wavelength of 485 nm and fluorescence emission intensity was measured at a wavelength of 528 nm. Standards were measured in triplicates and samples in duplicates.

4.10.7 Density gradient centrifugation of DNA and RNA

DNA and RNA stable isotope probing (SIP) were performed according to published protocols (Neufeld *et al.* 2007b, Whiteley *et al.* 2007, Degelmann *et al.* 2009). DNA was added

to a gradient solution (buoyant density 1.725 g mL^{-1}) containing a cesium chloride solution (buoyant density 1.881 g mL^{-1}; 80.8 % of total) and gradient buffer (pH 8.0; 100 mM Tris-HCl; 100 mM KCl; 1 mM EDTA; 19.2 % of total). RNA was added to a gradient solution (buoyant density of 1.796 g mL^{-1}) containing cesium trifluoroacetate (buoyant density 2.0 g mL^{-1}, 79.3 % of total), 3.1 % deionized formamide and gradient buffer (pH 8.0, 100 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 17.6 % of total). Gradient solutions and RNA or DNA were filled into OptiSeal Tubes (Beckmann, Fullerton, CA, USA). Differences within the gradient density could cause differences in the gene libraries prepared from gradient fractions, thus resulting in inconsistencies in determining which microorganisms are labeled. This problem was minimized by preparing all gradients with the same gradient solution.

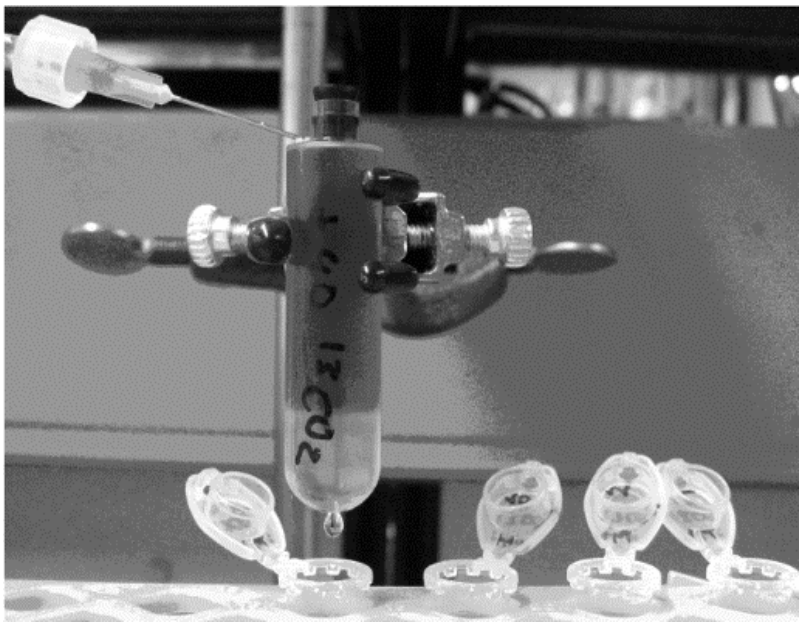


Figure 15: Fractionation of gradients after isopycnic centrifugation.

Image was taken by S. Hunger.

DNA was subjected to isopycnic centrifugation (177,000 *g* [44,100 rpm]) at 20 °C for 40 hours (VTi 65.2 vertical rotor, Beckman Coulter, Brea, USA) and was fractionated. RNA was subjected to isopycnic centrifugation (130,000 *g* [37,800 rpm]) at 20 °C for 67 hours (VTi 65.2 vertical rotor, Beckman Coulter, Brea, USA) and was fractionated. For fractionation of the gradients, blue dyed DEPC-treated water (4.4.20) was pumped (Econo Pump 1, Bio-Rad, Hercules, USA) with a flow rate of 455 µL min^{-1} into the centrifugation tubes (OptiSeal

Polyallomer centrifugation tubes, 13 x 48 mm, Beckmann Instruments, Brea, USA) pushing the gradient solution with the same flow rate from the centrifugation tubes into a new tube. Fractions of gradient solutions were collected in one-minute intervals.

The buoyant density of the DNA and RNA gradient solutions and fractions was determined by weighing gradient solutions and fractions at 20 °C and 25 °C, respectively, and a digital refractometer (Reichert-Analytical Instruments). DNA was precipitated with polyethylene glycol 6000 and glycogen (4.10.2), dissolved in 30 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and concentrations were measured with Quant-iT PicoGreen Assay Kit (4.10.6). RNA was precipitated with sodium acetate buffer (3 M, pH 5.2), glycogen and RNase-free 96 % ethanol (4.10.4), dissolved in 20 µL RNase-free deionized water, and concentrations measured with Quant-iT RiboGreen Assay Kit (4.10.6).

4.10.8 Reverse Transcription

RNA was transcribed into complementary DNA (Sambrook *et al.* 1989) with SuperScript III Reverse Transcriptase or SuperScript III First-Strand Synthesis Super Mix modified from the manufacturer's instructions (Invitrogen, Carlsbad, USA) (Table 9, Table 10).

Table 9: Reagents for first step of the reverse transcription.

Reagents	SuperScript III Reverse Transcript	SuperScript III First-Strand Synthesis SuperMix
Random primers	50 ng	50 ng
dNTPs[a] (10 mM each)	1 µL	-
Annealing buffer	-	1 µL
Template RNA	10 pg - 5 µg	0.1 pg - 5 µg
RNase-free water	ad 13 µL	ad 8 µL

[a] Legend: dNTP: deoxyribonucleotide.

The secondary structure of the RNA was disrupted by heating RNA and reagents included in step one of the reverse transcription (Table 9) to 65 °C for five minutes. Reagents were cooled on ice for one minute. Reagents for step two were added and homogenized (Table 10). An annealing step at 25 °C for five minutes allowed the random primers to bind

RNA. The complementary DNA strand was formed by the elongation of the primers performed by the reverse transcriptase at 50 °C for 50 and 120 minutes with reagents of SuperScript III First-Strand Synthesis SuperMix and SuperScript III Reverse Transcript, respectively. The reverse transcriptase was inactivated by heat treatment at 85 °C for five minutes and 70 °C for 15 minutes with reagents of SuperScript III First-Strand Synthesis SuperMix and SuperScript III Reverse Transcript, respectively. Complementary DNA was precipitated to remove reagents (4.10.3).

Table 10: Reagents for second step of the reverse transcription.

Reagents	SuperScript III Reverse Transcript	SuperScript III First-Strand Synthesis SuperMix
First-Strand Reaction Mix (2X)[a]	-	10 µL
First-Strand Buffer (5X)[b]	4 µL	-
DTT (0.1 M)[c]	1 µL	-
SuperScript III/RNaseOUT Enzyme Mix[d]	-	2 µL
SuperScript Reverse Transcriptase (200 U μL^{-1})	1 µL	-
RNase-free water	1 µL	-

[a] Includes 10 mM $MgCl_2$, 1 mM of each deoxyribonucleotide in buffer.
[b] Includes 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3.
[c] Dithiothreitol (DTT) was added to stabilize and improve the function of the reverse transcriptase.
[d] Includes a reverse transcriptase and an RNase inhibitor. Unit counts of enzymes are unknown.

4.10.9 Polymerase chain reaction (PCR)

One cycle of a polymerase chain reaction (PCR) is structured in three main steps (Table 12) (Sambrook *et al.* 1989): [1] denaturation of the template DNA, [2] annealing of primers (Table 11) on single stranded template DNA, and [3] elongation of primers by a DNA polymerase of *Thermus aquaticus* (*Taq* polymerase) (Chien *et al.* 1976, Saiki *et al.* 1988).

Table 11: Properties of primers.

Primer and target	Sequence	Reference
Bacterial 16S rRNA genes		
27f	5'-AGA GTT TGA TCM TGG CTC-3'	Lane 1991
907r	5'-CCG TCA ATT CMT TTR AGT-3'	
Methyl-CoM reductase-encoding genes, mcrA/mrtA		
mcrAf	5'-TAY GAY CAR ATH TGG YT-3'	Springer *et al.* 1995
mcrAr	5'-ACR TTC ATN GCR TAR TT-3'	
Formyltetrahydrofolate synthetase-encoding genes, fhs		
FTHFSf	5'-TTY ACW GGH GAY TTC CAT GC-3	Leaphart and Lovell 2001
FTHFSr	5'-GTA TTG DGT YTT RGC CAT ACA-3'	
Bacterial [FeFe]-hydrogenase-encoding genes		
HydH1f[a]	5'-TIA CIT SIT GYW SYC CIG SHT GG-3'	Schmidt *et al.* 2010; Schmidt *et al.* 2011
HydH3r	5'-CAI CCI YMI GGR CAI SNC AT-3'	
Bacterial group 4 [NiFe]-hydrogenase-encoding genes		
NiFe-uniF	5'-GAI MGI RTI TGY GGI ATH TGY-3'	Schmidt *et al.* 2011
NiFe-uniFb	5'-GAR MGI GTI TGY TCI CTG TGY-3'	
NiFe-uniR	5'-GTR CAI SWI WIR CAI GGR TC-3'	
pGEM-T vector-derived inserts		
M13f	5'-GTA AAA CGA CGG CCA G-3'	Messing 1983
M13r	5'-CAG GAA ACA GCT ATG ACC-3'	
pJET1.2/blunt vector-derived inserts		
pJET1.2f	5'-CGA CTC ACT ATA GGG AGA GCG GC-3'	manufacturers' instructions
pJET1.2r	5'- AAG AAC ATC GAT TTT CCA TGG CAG-3'	

[a] The primer originally published in Schmidt *et al.* (2010) was modified as described in Schmidt *et al.* (2011).

The general bacterial community was analyzed using the bacterial 16S rRNA (Table 11). Methanogens were identified by *mcrA* and *mrtA*. *mcrA* encodes the alpha-subunit of methyl-CoM reductase (isoenzyme I) that catalyzes the terminal step in methanogenesis and *mrtA* encodes the alpha-subunit of the isoenzyme II (Thauer 1998, Pihl *et al.* 1994). Acetogens were analyzed with *fhs* that encodes the formyltetrahydrofolate synthetase. This enzyme catalyzes the activation of formate with ATP and one of the first steps of the methyl branch in

the acetyl-CoA pathway (Drake *et al.* 2006, Ragsdale and Pierce 2008). Hydrogenase-containing microorganisms were analyzed with genes that encode group 4 [NiFe]-hydrogenases and [FeFe]-hydrogenases. DNA fragments were amplified with primers according to the vector plasmids used for cloning.

Table 12: Temperature and time protocols for PCR reactions.

Target	Temperature in °C/duration in minutes:seconds						
	Bacterial 16S[a]	*mcrA*/ *mrtA*[b]	*fhs*[c]	Genes for [FeFe]-H_2ase[de]	Genes for group 4 [NiFe]-H_2ase[fe]	Insert from vector plasmid[g]	Insert from vector plasmid[h]
Primer pair	27f, 907r	mcrAf, mcrAr	FTHFSf, FTHFSr	HydH1f, HydH3r	NiFe-uniF, NiFe-uniFb, NiFe-uniR	M13f, M13r	pJET1.2f, pJET1.2r
Initial denaturation	95/5:00	94/5:00	94/5:00	95/5:00	95/5:00	94/10:00	95/5:00
Denaturation I	95/1:00	94/0:45	94/0:30	95/0:45	95/0:45	94/0:45	94/0:30
Annealing I	40/1:00	50/0:45	58/0:45	55/0:45	50/0:45	54/0:45	60/0:30
Elongation I	72/1:00	72/0:45	72/1:10	72/1:30	72/1:30	72/3:00	72/1:30
Cycles I	5	35	35	40	40	30	35
Denaturation II	95/0:30	-	-	-	-	-	-
Annealing II	43/0:30	-	-	-	-	-	-
Elongation II	72/1:10	-	-	-	-	-	-
Cycles II	30	-	-	-	-	-	-
Terminal elongation	72/5:00	72/5:00	72/5:00	72/5:00	72/5:00	72/5:00	72/5:00

[a] Bacterial 16S rRNA genes were amplified according to Lane (1991).
[b] Methyl-CoM reductase-encoding genes were amplified modified from Lueders *et al.* (2001).
[c] Formyltetrahydrofolate synthetase-encoding gene were amplified modified from Leaphart and Lovell (2001).
[d] Bacterial [FeFe]-hydrogenase-encoding genes were amplified according to Schmidt *et al*. (2010) and Schmidt *et al.* (2011).
[e] Legend: H_2ase, hydrogenase.
[f] Bacterial group 4 [NiFe]-hydrogenase-encoding genes were amplified according to Schmidt *et al.* (2011).
[g] Inserts from pGEM-T vector were amplified according to Messing (1983).
[h] Inserts from pJET1.2/blunt vector were amplified according to manufacturer's instructions.

Protocols for temperature, time, and PCR reagents were adjusted for each PCR assay to obtain stringent PCR protocols (Table 12, Table 13). PCR assays were run in a

thermocycler (Labcycler, SensoQuest Biomedizinische Elektronik, Göttingen, Germany; peqSTAR 96 Universal, Peqlab Biotechnology, Erlangen, Germany).

Table 13: Protocols for PCR reagents.

Target	Final concentrations (unit listed with reagents)						
	Bacterial 16S[ab]	*mcrA*/ *mrtA*[ac]	*fhs*[ad]	Genes for [FeFe] H_2ase[aef]	Genes for group 4 [NiFe] H_2ase[afg]	Insert from vector plasmid[hi]	Insert from vector plasmid[ij]
Primer pair	27f, 907r	mcrAf, mcrAr	FTHFSf, FTHFSr	HydH1f, HydH3r	NiFe-uniF, NiFe-uniFb, NiFe-uniR	M13f, M13r	pJET1.2f, pJET1.2r
Each Primer (µM)	0.6	4	4	2	0.5	0.2	0.2
Bovine serum albumin (mg mL^{-1})	0.4	0.4	0.4	0.1	0.1	-	-
Each dNTP[k] (mM)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Taq DNA polymerase (U 25 μL^{-1})	0.6	0.6	0.6	0.6	0.6	1	1
$MgCl_2$ (mM)	3.6	2.6	3.6	3	3	2	2

[a] PCR assay was prepared with 5 Prime master mix.
[b] Bacterial 16S rRNA genes were amplified modified from Lane (1991).
[c] Methyl-CoM reductase-encoding genes were amplified modified from Lueders *et al.* (2001).
[d] Formyltetrahydrofolate synthetase-encoding genes were amplified modified from Leaphart and Lovell (2001).
[e] Bacterial [FeFe]-hydrogenase-encoding genes were amplified according to Schmidt *et al*. (2010) and Schmidt *et al.* (2011).
[f] Legend: H_2ase, hydrogenase.
[g] Bacterial group 4 [NiFe]-hydrogenase-encoding genes were amplified according to Schmidt *et al.* (2011).
[h] PCR assay was prepared with Crystal *Taq* polymerase and buffer B (final concentrations: 80 mM Tris HCl, pH 9.5, 20 mM $(NH_4)_2SO_4$, 0.02 % (w/v) Tween-20) (Biolab, Lüneburg, Germany).
[i] Inserts from pGEM-T vector were amplified according to Messing (1983).
[j] Inserts from pJET1.2/blunt vector were amplified according to manufacturer's instructions.
[k] Legend: dNTP: deoxyribonucleotide.

Negative and positive controls were prepared with each PCR. PCR-water (4.4.21) was used to prepare PCR assays and was also used as negative control to visualize potential contaminations. PCR assays were only utilized for further analysis if the negative control showed no signal on agarose gels (i.e., no contaminations). Chromosomal DNA of the

methanogen *Methanosarcina mazei* was used as positive control for analysis of methanogens. Chromosomal DNA of the acetogen *Thermoanaerobacter kivui* was used as positive control for PCR assays that targeted *Bacteria* in general and bacterial acetogens. Chromosomal DNA of *E. coli* and a fen-derived 'soil' sample were used as positive controls for PCR assays that target bacterial group 4 [NiFe]-hydrogenase-encoding genes and bacterial [FeFe]-hydrogenase-encoding genes, respectively.

4.10.10 Quantitative PCR

The quantity of a certain gene can be determined with quantitative PCR (qPCR). The procedure of qPCR is similar to PCR (4.10.9) with the addition of a quantification step of the target genes. A fluorescent dye such as SYBR Green is added to the PCR assay that intercalates with double stranded DNA (as specified by the manufacturer). The assay also contained another fluorescent dye named fluorescein that is used as an internal reference to normalize instrument and pipetting variations (as specified by the manufacturer). SYBR Green was excited at a wavelength of 490 nm and fluorescence emission intensity was measured at a wavelength of 530 nm. Thus, the increasing quantity of target gene is measured by the increasing fluorescence signal of SYBR Green. In this dissertation, bacterial 16S rRNA genes and *mcrA* were quantified to estimate the number of *Bacteria* and methanogen in environmental samples (Table 14, Table 15, Table 16), respectively.

Table 14: Properties of primers for qPCR.

Primer and target	Sequence	Reference
Bacterial 16S rRNA genes		
Eub341f	5'-CCT ACG GGA GGC AGC AG-3'	Muyzer *et al.* 1993
Eub534r	5'-ATT ACC GCG GCT GCT GG-3'	
Methyl-CoM reductase-encoding genes, mcrA/mrtA		
mcrAf	5'-TAY GAY CAR ATH TGG YT-3'	Springer *et al.* 1995
mcrAr	5'-ACR TTC ATN GCR TAR TT-3'	

'Soil' samples were taken 4-100 m apart from each other and were collected within nine days from all four mires

Three ‘soil’ samples per mire (4.1.1, 4.1.2, 4.1.3) were obtained for nucleic acid extractions (4.10.1). Each ‘soil’ sample was extracted three times, and each extraction was used for the triplicate qPCR assay for determining gene copy numbers. Samples were diluted 100-fold before adding to the qPCR reaction to minimize potential inhibition (Table 16).

Table 15: Temperature and time protocols for qPCR reactions.

	Temperature in °C/duration in minutes:seconds	
Target	Bacterial 16S[a]	*mcrA*/*mrtA*[b]
Primer pair	Eub341f, Eub534r	mcrAf, mcrAr
Initial denaturation	95/8:00	95/8:00
Denaturation	95/0:30	95/0:45
Annealing	55.7/0:40	62/0:45
Elongation[c]	72/0:30	72/0:45
Cycles	30	50
Terminal elongation	72/5:00	72/5:00

[a] Bacterial 16S rRNA genes were amplified modified from Muyzer *et al.* (1993).
[b] Methyl-CoM reductase-encoding genes were amplified modified from Depkat-Jakob *et al.* (2012).
[c] Fluorescence signal was monitored after elongation was completed.

qPCR assays were run in a qPCR cycler (iQ5 multicolor real-time PCR detection system, Bio-Rad Laboratories, Hercules, USA). Negative and positive controls were prepared with each qPCR. PCR-water (4.4.21) was used to prepare PCR assays and was also used as negative control to visualize potential contaminations. PCR assays were only utilized for further analysis if the negative control showed no signal on agarose gels (i.e., no contamination). Six standards of known template concentrations were prepared with each qPCR assay and copy numbers of target genes were calculated based on that standard curve. Melting curves were analyzed from 55-95 °C with increments of 0.5 °C.

Standards were prepared from pGEM vector or pJET1.2/blunt vector plasmid inserts of the target gene. For preparation of the insert, see chapter 4.10.14. The inserts were amplified with the primers M13f and M13r or pJET1.2f and pJET1.2r (4.10.9). Nucleic acids of PCR product were purified with an agarose gel (4.10.13), precipitated with isopropyl alcohol (4.10.3), resuspended in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and

quantified with Quant-iT dsDNA PicoGreen Assay Kit (4.10.6). The purified PCR product served as template for qPCR standards. Gene copy numbers of target genes in template DNA were calculated based on the concentration of template DNA (4.12.6).

Table 16: Protocols for qPCR reagents.

	Final concentrations (unit listed with reagents)[a]	
Target	Bacterial 16S[b]	*mcrA*/*mrtA*[c]
Primer pair	Eub341f, Eub534r	mcrAf, mcrAr
Each Primer (µM)	0.75	1.25
Bovine serum albumin (mg mL^{-1})	-	0.25
$MgCl_2$ (mM)	3	6
Template (%)	25	25

[a] Each PCR assay was prepared with SensiMix SYBR & Fluorescein Kit. Concentration of deoxyribonucleotides and heat-activated DNA polymerase in master mix is not given by the manufacturer.
[b] Bacterial 16S rRNA genes were amplified modified from Muyzer *et al.* (1993).
[c] Methyl-CoM reductase-encoding genes were amplified modified from Depkat-Jakob *et al.* (2012).

4.10.11 Purification of PCR products

DNA used for DNA SIP (4.10.7) was separated from RNA and purified from contaminants with Qiagen-tip Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Fifty-five microliter of sample containing nucleic acids were homogenized with 150 µL QRL1 buffer and 1.35 mL QRV2 buffer, and centrifuged (14,000 *g*, 4 °C, five minutes). A purification column was equilibrated with one milliliter QRE buffer. The sample-buffer mixture was transferred to the top of the column. The sample was pulled through the column by gravity and was collected (sample with DNA). The column was cleaned from RNA and contaminants such as proteins or polysaccharides with two milliliters QRW and one milliliter QRU buffer (45 °C). The sample with DNA was transferred to the column again and washed with three milliliters QC buffer. DNA was detached from the column with one milliliter QF buffer (45 °C) and collected. DNA was precipitated with isopropyl alcohol (4.10.3).

4.10.12 Agarose gel electrophoresis

Quality of PCR products was determined by agarose gel electrophoresis (gel electrophoresis apparatus, Techne, Jahnsdorf, Germany) (Sambrook *et al.* 1989). A one percent agarose gel was prepared with TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.5, AppliChem GmbH, Darmstadt, Germany) and 0.5 µg mL^{-1} ethidium bromide. Samples with nucleic acids were diluted 1:5 with gel loading dye and transferred in pockets of the agarose gel. Fragment lengths were determined with a molecular weight marker (MWM-1, 200-10,000 base pairs, Biovendis, Mannheim, Germany) that was also transferred in a pocket of the agarose gel. A voltage of 90 mV was applied to the agarose gel by a power supply (Power-Pak 3000, Bio-Rad Laboratories, Hercules, USA) for 20-50 minutes depending on the length of the fragment. Fragments in agarose gel were visualized with a Transilluminator (UVT-20M, Herolab, Wiesloch, Germany) and documented with a PowerShot G5 (Canon, Krefeld, Germany).

4.10.13 Purification of nucleic acids by agarose gel electrophoresis

DNA fragments for preparation of qPCR standards (4.10.10, 4.12.6) were purified by agarose gel electrophoresis to ensure that samples contained only fragments of a certain base pair length. Agarose gel electrophoresis was performed with a one percent agarose gel (4.10.12). The agarose gel was prepared with modified TAE buffer (40 mM Tris, 0.1 mM EDTA, pH 8.0, Merck Millipore, Darmstadt, Germany). DNA fragments of desired length were cut out of the agarose gel and purified with Montage DNA Gel Extraction Kit (Merck Millipore, Darmstadt, Germany) according to manufacturer's instructions. The gel slice was added to the gel nebulizer in the extraction device. The assembled device was centrifuged (10 minutes, 5,000 *g*). During this step, the agarose gel was nebulized, DNA and TAE buffer passed through a microporous membrane, and were collected at the bottom of the extraction device. The agarose was held back by the membrane. DNA in TAE buffer was precipitated with isopropyl alcohol (4.10.3) and dissolved in 30 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

4.10.14 Cloning

Cloning was performed modified from Sambrook *et al.* (1989). PCR products for cloning were ligated into pGEM vector plasmid (pGEM-T System, Promega, Mannheim, Germany, modified manufactures' protocol) or pJET1.2/blunt vector plasmid (CloneJET PCR Cloning Kit, Thermo Fisher Scientific, Waltham, USA, manufacturer's protocol). DNA in PCR products were separated from reagents of PCR reaction by precipitation of DNA with isopropyl alcohol (4.10.3). Precipitated DNA was dissolved in 30 µL deionized water before use for ligation.

For ligation of DNA fragments into pGEM-T vector plasmid, five microliters Rapid Ligation Buffer (2X), one microliter pGEM-T vector (50 ng), one microliter T4 DNA ligase (3 U μL^{-1}), one microliter template PCR product and two microliters deionized water were homogenized and incubated over night at 4 °C. The DNA fragment was ligated into the vector plasmid during this step.

For ligation into pJET1.2/blunt vector plasmid, 5 µL reaction buffer (2X, content unknown), 0.5 µL DNA blunting enzyme (U μL^{-1} unknown), 1 µL template PCR product, and 2.5 µL deionized water were homogenized, incubated at 70 °C for five minutes, and cooled on ice. During this step, 3'-overhangs were removed from the DNA fragment and 5'-overhangs were filled in. In a subsequent step, 0.5 µL pJET1.2/blunt cloning vector (50 ng μL^{-1}) and 0.5 µL T4 DNA ligase (5 U μL^{-1}) were added, homogenized, and incubated at room temperature for five minutes. The DNA fragment was ligated into the vector plasmid during this step.

Performed on ice, 50 µL competent cells of *E. coli* JM109 (manufacturer's instructions) were transformed with two microliter ligated vector plasmid. Competent cells and plasmids were incubated on ice for 30 minutes to allow vector plasmids to attach to outer permeable cell membranes. Vector plasmids were incorporated into cells during a 45-second heat shock at 42 °C (Thermomixer, Eppendorf, Madison, USA). Cells were cooled on ice and cautiously homogenized with 950 µL SOC medium (4.4.19). Heat shock treated cells in SOC medium

regenerated for 1.5 hours at 37 °C. Regenerated cells were harvested by centrifugation (5,000 *g*, six minutes). Eight hundred microliter of supernatant were discarded and the pellet was resolved in the leftover 200 µL. This cell suspension was transferred to LB agar plates (4.4.18). Only cells that incorporated a vector plasmid (both vectors included an Ampicillin resistance gene) formed single colonies on LB agar plates (4.4.18) over night at 37 °C. The pGEM-T vector plasmid additionally contained a *lac* operon (includes *lacZ* encoding for β-galactosidase) which becomes non-functional due to insertion of a DNA fragment during ligation (i.e., no functional β-galactosidase may be formed). Cells that contained an insertion in *lacZ* formed white colonies. Cells that contained a functional *lac* operon had no insertion of a DNA fragment and produced a functional β-galactosidase. Beta-galactosidase can be induced by IPTG and converts X-gal to 5-bromo-4-chloro-indoxyl that spontaneously forms 5,5'-dibromo-4,4'-dichloro-indigo a blue insoluble pigment. Those cells formed blue colonies. The pJET1.2/blunt vector plasmid contains the lethal gene *eco47IR*. Only cells with inserted DNA fragment do not experience the *eco47IR* lethality and form colonies. White colored colonies were picked randomly from LB agar plates.

Correct inserts were determined by M13 or pJET1.2 PCR (primer set M13f/M13r and pJET1.2f/pJET1.2r, 4.10.9) according to publication or manufacturer's instructions (Messing 1983), respectively. PCR fragments of selected clones were sequenced by Macrogen (Sanger method, Amsterdam, Netherlands).

4.11 Bioinformatics

4.11.1 Processing of sequences and assignment to phylotypes

All sequences were analyzed with Mega (Tamura *et al.* 2007) and ARB software (Ludwig *et al.* 2004). Mega software was used to remove residues of vectors and primers. MegaBLAST was used to compare sequences to those in public databases (Morgulis *et al*. 2008). Chimeric sequences of 16S rRNA gene sequences were identified by the greengenes tool Bellerophon (DeSantis *et al.* 2006) or by BLAST, and were excluded from further analysis.

Phylotypes of 16S rRNA and 16S rRNA genes were determined with RDP Classifier at a confidence threshold of 80 % (Wang *et al.* 2007), aligned with SINA web aligner (Pruesse *et al.* 2012), and merged with the latest 16S rRNA database from the SILVA homepage (www.arb-silva.de) (Pruesse *et al.* 2007). Sequences of 16S rRNA and 16S rRNA genes were assigned to novel species- and family-level phylotypes based on a similarity threshold of 97 % and 87.5 % (Yarza *et al.* 2008), respectively. Sequences of *mcrA*, *fhs*, and hydrogenase genes were translated *in silico* and aligned with reference sequences obtained from MegaBLAST using ClustalW algorithm implemented in ARB software. Assignment of *mcrA* and *fhs* sequences to species- and family-level phylotypes is based on similarity thresholds of (a) 85.7 % and 75.4 % for *mcrA* and (b) 76.4 % and 50.0 % for *fhs* (for details about the calculation see 4.11.5, 5.1.2, and Hunger *et al.* [2011a]).

ARB software was used to align sequences, create distance matrices with the neighbor joining method, and calculated phylogenic trees (4.11.3). The distance matrices were used to assign *mcrA*- and *fhs*-encoded amino acid sequences to species-level phylotypes with the software DOTUR (Schloss and Handelsman 2005). Nomenclature of affiliated microorganisms was determined with the homepage of “List of prokaryotic names with standing in nomenclature” (LPSN, http://www.bacterio.net). *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net).

4.11.2 Primer design

Primers targeting *fhs* (encodes the formyl-tetrahydrofolate synthetase) or *cooS* (encodes a CO dehydrogenase) were newly designed to assess information on acetogens. PCR conditions were optimized with genomic DNA of the acetogens *Terrisporobacter glycolicus* KHa (details about this isolate can be found in chapters 4.6.2 and 5.6), *Clostridium magnum* (DSM2767), *Clostridium drakei* (DSM12750), *Thermoanaerobacter kivui* (DSM2030), *Moorella thermoacetica* (DSM2955), *Moorella thermoautotrophica* (DSM1974), *Sporomusa silvacetica* (DSM10669), and *Acetobacterium woodii* (DSM1030).

4.11.3 Phylogenic trees

Phylogenic trees of *mcrA*, *fhs*, *cooS*, and hydrogenase genes were calculated with nucleotide and amino acid sequence with the following algorithms: neighbor-joining (Felsenstein, PAM, Olsen, or Dayhoff correction) (Saitou and Nei 1987), maximum-likelihood (Jukes-Cantor or Dayhoff correction) (Knoop and Müller 2009), and maximum-parsimony (no correction) (Knoop and Müller 2009). Phylogenic trees of 16S rRNA and 16S rRNA genes were calculated with nucleotide sequences with the following algorithms: neighbor-joining (felsenstein correction) (Saitou and Nei 1987), maximum-likelihood (new rapid hill climbing algorithm) (Knoop and Müller 2009), and maximum-parsimony (no correction) (Knoop and Müller 2009). More details concerning the calculation of trees can be found in the legend of the corresponding tree.

4.11.4 Rarefaction analysis

Rarefaction curves show the calculated number of phylotypes after n sequences (Hurlbert 1971, Heck *et al.* 1975) and allow estimations on the number of phylotypes depending on the number of sequences obtained. A flattening curve indicates that only a few more phylotypes were to be expected if more sequences were obtained and that sequencing was sufficient.

4.11.5 Similarity plots

Species- and family-level threshold values were determined for the structural genes *fhs* and *mcrA* based on species- and family-level threshold values of 16S rRNA sequences (modified from Palmer *et al.* 2009).

Sequences of structural genes and 16S rRNA from the same microorganism was obtained from NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and SILVA (http://www.arb-silva.de), respectively. Structural genes were *in silico* translated into amino acid sequences and aligned with ARB software. The 16S rRNA sequences were aligned by SINA web aligner (Pruesse *et al.* 2012) prior to

download. Similarities in the sequences of different microorganisms were calculated by a pairwise comparison with MEGA software. Similarities (S) were calculated from the difference between two nucleotide or amino acid sequences (D with 0 ≤ D ≤ 1):

$$S = 1 - D$$

Phylogenic correlation plots (Purkhold *et al.* 2001, Palmer *et al.* 2009) of 16S rRNA gene sequence similarities and amino acid sequence similarities of *mcrA* or *fhs* were prepared with the following filters: for *mcrA*, 131 valid amino acids between positions 98 and 227 of *mcrA*-encoded amino acid sequence of *Methanocella paludicola* SANAE (NC_009089); for *fhs*, 351 valid amino acids between positions 134 and 486 of *fhs*- encoded amino acid sequence of *Clostridium difficile* 630 (NC_009089). Similarities of *mcrA*- or *fhs*-encoded amino acid sequences were plotted against the similarities of the corresponding 16S rRNA sequences of the same microorganisms. In total, 79 *mcrA* and 16S rRNA sequences were used to determine threshold values for *mcrA*-encoded amino acids, and 235 *fhs* and 16S rRNA sequences were used to determine threshold values for bacterial *fhs*-encoded amino acids. Assignment of *mcrA* and *fhs* sequences to taxonomic hierarchic phylotypes was based on correlations between amino acid sequences of the translated structural gene to the 16S rRNA gene sequences of the same cultured microorganisms. 16S rRNA gene sequence similarities of 97.0 % and 87.5 % are conservative threshold values for determining species- and family-level differences, respectively, between microorganisms (Yarza *et al.* 2008).

4.12 Calculations and statistics

4.12.1 Calculation of recovery of carbon and reductant from supplemented substrates

The recovery of supplement-derived carbon and reductant in products was calculated to estimate the turnover of supplements. The calculation was based on the concentrations of supplements and the concentrations of observed products in the unsupplemented controls and the substrate-supplemented slurries within a certain time interval. The net concentrations of a

certain compound were determined by (A) subtracting the concentration of the unsupplemented control from the concentration of the substrate-supplemented slurries, and (B) subsequently subtracting the concentration at an earlier time point of the incubation from the concentration at a later time point of the incubation (e.g., the beginning and the end of substrate-utilization). For example, 5 mM glucose were fermented within five days, and 0.8 mM propionate, 4 mM acetate, 1.5 mM butyrate, 2 mM formate, 7 mM CO_2, and 12 mM H_2 more were detected in the glucose-supplemented slurries than in the unsupplemented control within those five days.

For the calculation of carbon recovery, the number of carbon atoms in a compound was multiplied by the net concentration of this compound yielding carbon-moles of available carbon. For example, five millimolar glucose was multiplied by six yielding 30 mM carbon-moles. The carbon-moles of the utilized substrate represent 100 % and carbon-moles of products were set in relation to that 100 % of the substrate. For the calculation of the recovery of reductant, the number of electrons was calculated that is needed to completely oxidize a certain compound. The number of electrons was multiplied by the net concentration of this compound yielding electron-moles of available reductant. For example, five millimolar glucose were multiplied by 24 yielding 120 mM electron-moles. The electron-moles of the utilized substrate represent 100 % and electron-moles of products were set in relation to that 100 % of the substrate. The addition of the percentages of products represents the recovery of either reductants or carbon from the utilized substrate. In the example mentioned above, 85 % of glucose-derived carbon were recovered (8 % in propionate, 27 % in acetate, 20 % in butyrate, 7 % in formate, 23 % in CO_2) and 84 % of glucose-derived reductant were recovered (9 % in propionate, 27 % in acetate, 25 % in butyrate, 3 % in formate, 20 % in H_2).

4.12.2 Mean value

The sample mean ($\bar{x}$) is calculated from the size of the sampling (n) and the sum of observed values of the sample ($\bar{x}_1$, $\bar{x}_2$, …) (Paulson 2008):

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

4.12.3 Standard deviation

The standard deviation (s) is calculated from the observed values of the sample ($\bar{x}_1$, $\bar{x}_2$, …), the mean value of all samples ($\bar{x}$), and the size of the sampling (n) (Paulson 2008):

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}}$$

4.12.4 Molarities in solutions and slurries

Solutions with a defined concentration were prepared for supplementation of slurries. The amount of compound (m in g) needed to prepare this solution was calculated from the desired concentration of this compound (c in mol L^{-1}) in solvent (i.e., water), the molar mass of this compound (M in g mol^{-1}), and the volume of the solvent (V in L):

$$m = cMV$$

Slurries were treated with a defined volume of stock solution (V_S in mL) of a desired compound. This volume was determined from the concentration of the stock solution (c_{St} in mM), the desired concentration in slurry (c_S in mM), and the total volume of the slurry (V_T in mL):

$$V_S = \frac{V_T c_S}{c_{St}}$$

4.12.5 Ideal gas law

The concentrations of gases in incubation flasks were calculated based on the ideal gas law (Blachnik 1998, Krichevsky and Kasarnovsky 1935):

$$p\,V_G = n_G\,R\,T$$

$$n_G = \frac{p\,V_G}{R\,T}$$

The ideal gas law considers the partial pressure of the gas (p in mbar), volume of the gas phase (V_G in mL), universal gas constant (R equals 83.145 mbar mL K^{-1} $mmol^{-1}$), and the actual temperature (T in K) for the calculation of the amount of gas in the gas phase (n_G in mmol).

For calculation of gas concentrations in incubation flasks, the partial pressure of a gas is calculated from the overpressure in the incubation flask (p_O in mbar), the actual atmospheric pressure (p_A in mbar), and the measured rate of the gas in the incubation flask (X in 10^{-2} %):

$$p = X\,(\,p_O\, +\, p_A)$$

The amount of physically dissolved gas in the liquid phase (n_L in mmol) was calculated from the partial pressure of the gas, volume of the liquid phase (V_L in mL), universal gas constant, the actual temperature, and the solubility coefficient (λ in Ncm^3[gas] g[H_2O]$^{-1}$, Table 17):

$$n_L = \frac{p\,V_L\lambda}{R\,T}$$

The amount of chemically dissolved CO_2 in form of bicarbonate in the liquid phase (n_C in mmol) was calculated from the amount of physically dissolved CO_2 in the liquid phase (n_L in mmol), the pH of the liquid phase, and the logarithmic acid dissociation constant for bicarbonate (pKa equals 6.37 at 25 °C):

$$n_C = \, n_L\, 10^{pH-pKa}$$

The total amount of gas (n_T in mmol) in an incubation flask was the sum of the amounts of gas in gas and liquid phase:

$$n_T = \, n_G + \, n_L + \, n_C$$

The total amount of gas (n_T) was used to calculate concentrations of gases per liter liquid phase, per gram dry weight, or per gram fresh weight.

Table 17: Solubility coefficient λ for gases dissolved in water at room temperature.

	Solubility coefficient λ in water (Ncm^3[gas] g[H_2O]$^{-1}$)[ab]			
	10 °C	15 °C	25 °C	30 °C
CO_2	0.987	0.851	0.646	0.516
CH_4	0.040	0.036	0.029	0.027
H_2	0.019	0.018	0.017	0.017
O_2	0.037	0.033	0.027	0.025

[a] Reference: Blachnik (1998).
[b] Ncm^3[gas] g[H_2O]$^{-1}$ describes the amount of gas in Ncm^3 that is dissolved in one gram water at 980.1 hPa.

4.12.6 Preparation of standards for qPCR

The quantity of a target gene (c_{TG} in targets µL^{-1}) was calculated taking into consideration the concentration of standard DNA (c_{ST} in ng_{DNA} µL^{-1}), length of the target gene (n_{TG} in base pairs), molecular weight of one base pair in water (MW_{bp}: 660 x 10^9 ng mol^{-1}), and the Avogadro constant (N_A: 6.23 x 10^{23} molecules mol^{-1}):

$$c_{TG} = \frac{c_{ST}}{n_{TG} \times MW_{bp}} \times N_A$$

The exact number of bases for each target gene was determined by Sanger sequencing (Macrogen, Amsterdam, Netherlands).

4.12.7 Conversion of rotational speed to relative centrifugal force

The relative centrifugal force (RCF in g) of a rotor is calculated from the rotational speed (N in revolution per minute, rpm), and the rotational radius (r in mm):

$$RCF = 1.12 \times 10^{-6} \times r \times N^2$$

4.12.8 Principal component analysis

The principal component analysis (PCA) was used to visualize a complex dataset in a single graph (Pearson 1901, Borg and Groenen 2005, Abdi and Williams 2010) and was performed with the software ‘R’. PCA is a multivariate statistical analysis that structures and simplifies a complex dataset and plots the dataset in response to two principal components

(PC1 and PC2) whereby the highest variance is shown by PC1 and the second highest by PC2. An arrow parallel to one of the axes of the principal components indicates the main driver of this variance. The following parameters of 'soils' (A) and pore water (B) were used to calculate the PCA plot for the comparison of contrasting mire 'soils': (A) relative abundance of phyla-level 16S rRNA gene phylotypes prior to treatments, relative abundance of family-level *mcrA* phylotypes prior to treatments, water content, total carbon, total nitrogen, C/N ratio, cultivable number of microorganisms under aerobic conditions, cultivable number of microorganisms under anaerobic conditions, abundance of *mcrA* gene copy numbers, and abundance of 16S rRNA gene copy numbers, and (B) pH, NPOC, concentrations of ions (Cl^-, NO_3^-, PO_4^{3-}).

4.12.9 Analysis of variance

The analysis of variance (ANOVA) was used to estimate significant differences between more than two sample groups (Paulson 2008). ANOVA compared the variance between sampling groups to the variance within sampling groups. The software 'R' and the library 'laercio' was used for ANOVA.

4.12.10 Coverage of sequencing

The coverage of sequencing was calculated to estimate if sampling was sufficient. The coverage of a certain gene library (C in %) was calculated from the number of phylotypes that contained a single sequence (n) and the total number of sequences obtained (N) (Schloss *et al.* 2004):

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

4.12.11 Change of Gibbs free energy

Gibbs free energy was calculated to estimate if a certain microbial process was thermodynamically feasible under the experimental conditions and to estimate the changing thermodynamics of simultaneously occurring processes. The change of the Gibbs free energy

(ΔG in kJ mol^{-1}) was calculated from the change of Gibbs free energy under standard conditions ($\Delta G°'$ in kJ mol^{-1}), actual temperature (T in K), gas constant (R: 8.31 J mL^{-1} K^{-1}), and equilibrium constant (K' without dimension) (Thauer *et al.* 1977, Conrad and Wetter 1990, Berg *et al.* 2003):

$$\Delta G = \Delta G°' + RTlnK'$$

The following equations were used to calculate Gibbs free energy values:

4 H_2 + CO_2 → CH_4 + 2 H_2O

4 H_2 + 2 CO_2 → CH_3COO^- + H^+ + 2 H_2O

4 $HCOO^-$ + 4 H^+ → CH_3COO^- + H^+ + 2 CO_2 + 2 H_2O

4 $HCOO^-$ + 4 H^+ → CH_4 + 3 CO_2 + 2 H_2O

$HCOO^-$ + H^+ → H_2 + CO_2

$C_6H_{12}O_6$ → 3 CH_3COO^- + 3 H^+

The Gibbs free energy under standard conditions was calculated from the standard Gibbs energy of formation ($G_f°$ in kJ mol^{-1}, Table 18) of substrates and products of interest:

$$\Delta G°' = \sum G_f°\,(products) - \sum G_f°\,(substrates)$$

Table 18: Gibbs energies of formation (∆Gf°)[a].

	∆Gf° (kJ mol^{-1})
Formate	-351.0
Acetate	-369.4
CO_2	-394.4
Water	-237.2
H^+ per pH value	-5.7
CH_4	-50.7
H_2	0.0

[a] Values derived from Thauer *et al.* (1977) and Madigan and Martinko (2006).

The equilibrium constant was calculated from actual concentrations of reactants ($[A]^a[B]^b$ in M or atm) and products ($[C]^c[D]^d$ in M or atm). Concentrations serve as base (i.e.,

A, B, C, D) and the stoichiometric number of reactants and products in the biochemical reaction serve as exponent (i.e., , a, b, c, d):

$$a\text{A} + b\text{B} \leftrightarrow c\text{C} + d\text{D}$$

$$K' = \frac{[C]^c[D]^d}{[A]^a[B]^b}$$

Values are given in kJ mol^{-1} CH_4 for methanogenesis, kJ mol^{-1} acetate for acetogenesis, and kJ mol^{-1} H_2 for conversion of formate to H_2 and CO_2.

4.13 Frequently used equipment

The following equipment was used frequently:

1.5 or 2.0 mL tubes (Safe Lock Tubes, Eppendorf, Hamburg, Germany); 1.5 or 2.0 mL tubes (Hartenstein, Würzburg/Versbach, Germany); 0.2 mL Top-line-PCR tubes (AHN Biotechnology, Nordhausen, Germany); 96 well plates (Sorenson Bioscience, Murray, USA); qPCR 96 well plates (Thermosprint plate 96, Biovendis, Mannheim, Germany); sterile filter (Minisart syringe filter, 0.2 µm pore size, Sartorius Stedim, Göttingen, Germany); filter (Nylon, 0.22 µm pore size, Merz Brothers, Haid, Austria); syringes (1 mL/3 mL/50 mL, Becton Dickinson, Madrid/Laagstraat/Oxford, Spain/Belgium/UK, respectively); 27G 3/4'' und 19G 1'' needle (0.4 x 19 mm/1.1 x 25 mm, Becton Dickinson, Drogheda, Ireland); 21G 4 3/4'' needle (0.8 x 120 mm, Becton Dickinson, Melsungen, Germany); serum bottles (Merck ABS, Dietikon, Switzerland); infusion flask (Müller + Krempel, Bülach, Switzerland); centrifuge 5415c (Eppendorf, Hamburg, Germany); centrifuge Sigma 1 15 K (Sigma-Aldrich, St. Louis, USA); Analytic AC 120 S weighing scale (Sartorius, Göttingen, Germany).

4.14 Chemicals, reagents and gases

Chemicals and reagents used in this study derived from Sigma-Aldrich (St. Louis, USA), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, USA), Campro Scientific (Berlin, Germany), 5 Prime (Hilden, Germany), Biomers (Ulm, Germany), Axon

Labortechnik (Kaiserslautern, Germany), Eppendorf (Hamburg, Germany), New England Biolabs (Ipswich, USA), Bio-Rad (Hercules, USA), Promega (Mannheim, Germany), Bioline (Luckenwalde, Germany), Microsynth (Balgach, Switzerland).

Gases derived from Rießner-Gase & Co. (Lichtenfels, Germany) (Table 19).

Table 19: Purity of utilized gases.

	Compressed air	Ar	N_2	H_2	CO_2	CH_4	He
Purity	DIN 3188	4.8	5.0	5.0	4.5	technical	4.6

4.15 Software

MPN values were calculated with the 'MPN Calculator' (www.i2workout.com/ mcuriale/mpn/index.html). Data obtained from gas chromatographs were recorded and processed with 'PeakSimple' (Chromatography Acquisition and Integration Software, SRI Instruments, Torrance, USA) or 'EuroChrom' (Acquisition and Integration Software, Knauer, Berlin, Germany). Data obtained from high performance liquid chromatography were recorded and processed with 'ChemStation' (Agilent Technologies, Santa Clara, USA). 'Gen5' was used to record and process data from the fluorimeter and spectrometer (BioTek Instruments, Winooski, USA). 'Remote Capture' was used to document the quality and fragment length of nucleic acids in agarose gels (Canon, Tokyo, Japan). 'MRGrab' was used to document cell morphologies of microorganisms (Carl Zeiss, Oberkochen, Germany). 'Optical System Software' was used to record and process data from iQ5 multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, USA). 'DOTUR' was used to assign sequences to phylotypes (https://github.com/mothur/DOTUR). 'R' was used for statistical analysis (https://www.r-project.org and https://www.rstudio.com). 'aRarefactWin' was used to calculate rarefaction curves (http://strata.uga.edu/ software/win/aRarefactWin.exe). 'Microsoft 2013' or 'Microsoft 2016' (Microsoft, Redmond, USA) and 'SigmaPlot' were used for processing, evaluation and visualization of data (Systat Software, San Jose, USA).

4.16 Accession numbers

Most sequences obtained in this study are available from EMBL nucleotide sequence database (Table 20).

Table 20: Accession numbers of sequences obtained from this study outlined in this dissertation.

Origin	Target	Accession number
Mire 'soils'	*mcrA*/*mrtA*	LN716108-7036
C. rostrata roots	*mcrA*/*mrtA*	LT009513-676
Substrate and gut contents of *E. eugeniae*	*mcrA*/*mrtA*	HE647204-384
Gut-derived methanogenic enrichment, *E. eugeniae*	*mcrA*/*mrtA*	LK936462-502
Glucose-supplemented gut contents of *E. eugeniae* (SIP)	Bacterial 16S rRNA	Not submitted
Mire 'soils'	Bacterial 16S rRNA gene	LN715239-6107
Root-derived enrichments	Bacterial 16S rRNA gene	LT009679-85
Root-derived isolates	Bacterial 16S rRNA gene	LT009677-8
Terrisporobacter glycolicus KHa	Bacterial 16S rRNA gene	FR850057
Bacteroides xylanolyticus KHb	Bacterial 16S rRNA gene	FR850058
Root-derived isolates	Hydrogenase gene	LT009686-7
Glucose-supplemented gut contents of *E. eugeniae* (SIP)	*fhs*	Not submitted
Formate-supplemented mire 'soil' (SIP)	*fhs*	FR725862-930
Terrisporobacter glycolicus RD-1	*fhs*	FR850046
Clostridium drakei DSM12750	*fhs*	FR850047
Moorella thermoautotrophica DSM1974	*fhs*	FR850048
Sporomusa silvacetica DSM10669	*fhs*	FR850049
Terrisporobacter glycolicus KHa	*fhs*	FR850050
Acetobacterium woodii DSM1030	*cooS*	FR850051
Thermoanaerobacter kivui ATCC33488	*cooS*	FR850052
Clostridium magnum	*cooS*	FR850053
Clostridium drakei DSM12750	*cooS*	FR850054
Terrisporobacter glycolicus RD-1	*cooS*	FR850055
Terrisporobacter glycolicus KHa	*cooS*	FR850056

4.17 Contribution of other persons to this dissertation

If not mentioned otherwise, sampling, preparation and processing of experiments, evaluation and visualization of data were performed by myself. The majority of the results were published in peer-review journals (Hunger *et al.* 2011a, Hunger *et al.* 2011b, Depkat-Jakob *et al.* 2012, Schulz *et al.* 2015, Hunger *et al.* 2015, Hunger *et al.* 2016) and were presented and discussed in this dissertation in a similar manner as in these publications. In addition, some of the findings constitute the basis of additional manuscripts that are in preparation.

4.17.1 Analysis of functional redundancy in contrasting mire 'soils'

The objective for this study and the experimental set-up were developed by myself. Part of the experiments were conducted by Anita S. Gößner (technician at the Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany) or Claudia Burger as part of her Bachelor thesis (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany). Both coworkers were supervised by myself. Sampling was performed by myself together with Anita S. Gößner and Claudia Burger. Preparation and processing of 'soil' slurries from mires 1-3 (4.5.1, 4.5.2, 4.8.7, 4.8.8) was performed by Anita S. Gößner. Extraction of DNA (4.10.1), PCR (4.10.9), picking of clones (4.10.14), and reamplifying ligated fragments (4.10.9) was performed by Anita S. Gößner. Ligation (4.10.14), transformation (4.10.14), and sequence analysis (4.11) was performed by myself. Preparation and processing of 'soil' slurries from mire 4 (4.5.1, 4.5.2) were performed by Claudia Burger. Extraction of DNA (4.10.1), PCR (4.10.9), and cloning (4.10.14) for samples before treatment was performed by Claudia Burger. Samples after treatment were processed by Anita S. Gößner and myself as described above. Analysis of cultivable microorganisms (4.7) and gene copy numbers (4.10.10) was performed by myself. Total nitrogen, total carbon, NPOC, and concentration of ions were analyzed at the Center for Analytical Chemistry (Bayreuth Centre of Ecological and Environmental Research, University of Bayreuth, Bayreuth, Germany). Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.2 Analysis of FHL activity, methanogenesis and acetogenesis with roots of mire-derived plants

The objective for this study and the experimental set-up were developed by myself. Part of the experiments were conducted by Anita S. Gößner, Madena Eppendorfer as part of her Master thesis (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany). Anita S. Gößner and Madena Eppendorfer were supervised by myself. Sampling was performed by myself together with Anita S. Gößner and Dr. Pedro Gerstberger (Plant Ecology Group, University of Bayreuth, Bayreuth, Germany). Dr. Pedro Gerstberger identified the plants. Preparation and processing of soil and root slurries (4.5.1, 4.8.7, 4.8.8) were performed by Anita S. Gößner and Madena Eppendorfer together with myself. Extraction of DNA (4.10.1), PCR (4.10.9), picking of clones (4.10.14), and reamplifying ligated fragments (4.10.9) was performed by Anita S. Gößner. Ligation (4.10.14), transformation (4.10.14), and sequence analysis (4.11) was performed by myself. Changes of Gibbs free energy were calculated by Oliver Schmidt and graphical presentation was performed by myself. Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.3 'Stable isotope probing' of mire 'soil' with [^{13}C]formate

Sampling, preparation and processing of experiments, DNA SIP, molecular analyzes of *mcrA* and 16S rRNA gene sequences, evaluation and visualization of process and molecular data were performed by myself as part of my diploma thesis. It was part of this doctoral dissertation to (a) analyze active acetogens by obtaining bacterial *fhs* sequences (2.10.9, 2.11.1) from those 'soil' slurries and (b) determine the content of ^{13}C-enriched acetate (2.8.9). Peter Claus and Prof. Dr. Ralf Conrad (Max Planck Institute of Terrestrial Microbiology, Marburg, Germany) conducted the analysis of ^{13}C-enriched organic acids and gases (4.8.9). Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.4 'Stable isotope probing' of gut contents derived from the earthworm *E. eugeniae* with [^{13}C]glucose

The objective for this study and the experimental set-up were developed by myself. Parts of the experiments were conducted by Kristin Schulz (Master student, Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany), Christina Bruß as part of her Master thesis (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany), Peter Claus (Max Planck Institute of Terrestrial Microbiology, Marburg, Germany), Prof. Dr. Ralf Conrad (Max Planck Institute of Terrestrial Microbiology, Marburg, Germany), and Prof. Dr. Harold L. Drake (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany). Sampling of earthworms and obtaining gut contents was performed by Kristin Schulz and Prof. Dr. Harold L. Drake. Preparation and processing of slurries with gut contents (4.2, 4.5.1, 4.8.7, 4.8.8) was performed by myself. Molecular analysis (4.10.1, 4.10.5, 4.10.7, 4.10.8, 4.10.9, 4.10.14, 4.11.1) was conducted by Christina Bruß. Christina Bruß was supervised by myself. Peter Claus and Prof. Dr. Ralf Conrad conducted the analysis of ^{13}C-enriched organic acids and gases (4.8.9). Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.5 Enrichment and isolation of FHL-containing bacteria, fermenters, and an acetogen from mire-derived roots

The objective for this study and the experimental set-up were developed by myself. Experiments were predominantly conducted by Anita S. Gößner who was elaborated and supervised by myself. Sampling of roots was performed by myself together with Anita S. Gößner. Preparation and processing of enrichments and characterization of isolates (4.6.1, 4.9) was predominantly performed by Anita S. Gößner and partly by myself. Extraction of DNA (4.10.1), PCR (4.10.9), and picking of clones (4.10.14) was performed by Anita S. Gößner. Ligation (4.10.14), transformation (4.10.14), and sequence analysis (4.11) of the acetogenic enrichment was performed by myself. Kristina Schraml analyzed the hydrogenase genes of isolates (4.10.9, 4.11.3) as part of a six week practical course (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany) and was elaborated and supervised

by Oliver Schmidt (doctoral student, Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany). Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.6 Isolation of an acetogen and a fermenter from forest 'soil'

The objective for this study and the experimental set-up were developed by Prof. Dr. Harold L. Drake and myself. Samples were taken by Prof. Dr. Harold L. Drake. Experiments were predominantly conducted by Anita S. Gößner who was predominantly supervised by Prof. Dr. Harold L. Drake. Molecular analysis (4.10.1, 4.10.9) and xylan-dependent product profiles were conducted by myself and Anita S. Gößner. Evaluation, graphical presentation, and interpretation of data were performed by myself.

4.17.7 Enrichment of methanogens and acetogens from gut contents of *E. eugeniae*

The objective for this study and the experimental setup were developed by myself. Sampling of earthworms and obtaining gut contents was performed by Dr. Peter S. Depkat-Jakob (Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany). Initial incubations were performed by myself and Dr. Peter Depkat-Jakob. Later incubations, enrichment, isolation, and characterization was conducted by myself. Evaluation, graphical presentation, and interpretation of data were performed by myself.

5 Results

5.1 Molecular tools for identification of methanogens and acetogens

5.1.1 Newly developed primers targeting acetogens

Acetogens are widespread within the *Bacteria* (Drake *at al.* 2006) and thus difficult to target by molecular approaches. The most common primers to target acetogens are primers that target *fhs* (FTHFSf and FTHFSr, Leaphart and Lovell 2001). *fhs* encodes the formyltetrahydrofolate synthetase (Leaphart and Lovell 2001), an enzyme of the acetyl-CoA pathway of acetogens binding the formyl group (-CHO) of formate to tetrahydrofolate forming formyltetrahydrofolate at the expense of one ATP (Drake *et al.* 2006, Ragsdale and Pierce 2008). Formyltetrahydrofolate synthetase is not restricted to acetogens and can also be found in other taxa such as sulfate-reducing bacteria, aceticlastic methanogens, and syntrophic acetate-oxidizing bacteria (Müller *et al.* 2013). Thus, *fhs* primers target acetogens and non-acetogens. The primers FTHFSf and FTHFSr target acetogens within the genera *Moorella* and *Sporomusa* very weakly (own experience). At the time of study, no other primers for the analysis of acetogens were available. Only a few acetogen-associated gene sequences that encode other enzymes of the acetyl-CoA pathway were available from NCBI (i.e., 2009 and 2010). For example, *cooS* encodes a carbon monoxide dehydrogenase that can be a part of the CODH/ACS complex of acetogens (González and Robb 2000, Ragsdale and Pierce 2008, Köpke *et al.* 2013). *CooS* sequences of the acetogens *Clostridium difficile* (AM180355) and *Blautia hydrogenotrophica* (NC_ACBZ01000127) were available but both sequences clustered more closely to non-acetogens than to each other in phylogenetic trees (Figure 16). It was expected that samples of interest for molecular analysis contained *Clostridium*-related species rather than *Blautia*- and *Moorella*-related species and thus *cooS* primers for *Clostridium*-related species were designed (Table 21). Also, new primers for *fhs* were designed that target *Moorella* and *Sporomusa*.

Table 21: Newly designed primers for *fhs* and *cooS*.

Primer and target	Sequence
Formyltetrahydrofolate synthetase-encoding genes, fhs	
fhs610f[a]	5'-GTW GCH TCI GAR RTI ATG GC-3'
fhs1249r[a]	5'-CYR CCY TTH GCC CAN AC-3'
Carbon monoxide dehydrogenase-encoding genes, cooS	
cooS805f[a]	5'-AAR SCM CAR TGT GGT TTT GG-3'
cooS2623rw[a]	5'-TTT TST KMC ATC CAY TCT GG-3'
cooS103f-I[b]	5'-AAG RCA CMD TGT GGT TTT GG-3'
cooS103f-II[b]	5'-ACW CCG CAC TGT AAA TTT GG-3'
cooS634r[b]	5'-GAA DCC IVC CAA ICC RTC-3'
cooS896r-I[b]	5'-GCC AWT TTW RYR CCA TGT C-3'
cooS896r-II[b]	5'-CAT SGG RAT TCC SCK KC-3'

[a] Published in Hunger *et al.* (2011b).
[b] Unpublished.

Temperature and time protocols, concentrations of primers and concentrations of magnesium were optimized with genomic DNA from the acetogen *Thermoanaerobacter kivui* (positive control) and genomic DNA from *Methanosarcina mazei* (negative control) for each assay (Table 22, Table 23).

Table 22: Temperature and time protocols for newly designed *fhs* and *cooS* primers.

	Temperature in °C/duration in minutes:seconds			
	fhs610f & fhs1249r	cooS805f & cooS2623rw	cooS103f-I, cooS103f-II & cooS634r	cooS103f-I, cooS103f-II & cooS896r-I, cooS896r-II
Initial denaturation	94/5:00	94/5:00	94/5:00	94/5:00
Denaturation	94/0:45	94/0:45	94/0:45	94/0:45
Annealing	56/0:45	55/0:45	59/0:45	56/0:45
Elongation	72/1:00	72/2:20	72/1:00	72/1:10
Cycles	35	45	45	45
Terminal elongation	72/5:00	72/5:00	72/5:00	72/5:00

The primer fhs610f and fhs1249r showed a signal of approximately 640 base pairs and targeted the acetogens *Thermoanaerobacter kivui*, *Moorella thermoacetica*, *Moorella*

thermoautotrophica, *Clostridium drakei*, *Clostridium magnum*, *Terrisporobacter glycolicus*, *Sporomusa silvacetica*, and *Acetobacterium woodii* but not the non-acetogen *Methanosarcina mazei*. *fhs* sequences of *T. glycolicus* RD-1, *C. drakei*, *M. thermoacetica*, and *S. silvacetica* were not publicly available and were sequenced and submitted to EMBL database (Table 20).

Table 23: Protocols for PCR reagents of newly designed *fhs* and *cooS* primers.

Reagents	Concentrations (unit listed with reagents)[a]			
	fhs610f & fhs1249r	cooS805f & cooS2623rw	cooS103f-I, cooS103f-II & cooS634r[b]	cooS103f-I, cooS103f-II & cooS896r-I, cooS896r-II
Each Primer (µM)	4	4	1	1
Bovine serum albumin (mg mL^{-1})	0.4	0.4	0.4	0.4
Each dNTP[b] (mM)	0.2	0.2	0.2	0.2
Taq DNA polymerase (U μL^{-1})	0.24	0.24	0.24	0.24
$MgCl_2$ (mM)	2.6	2.6	2.6	2.6

[a] Each PCR assay was prepared with 5 Prime master mix.
[b] Legend: dNTP: deoxyribonucleotide.

The alignment of *cooS* sequences was diverse and primers could not be designed for all *cooS* sequences. The primers cooS805f and cooS2623rw were designed with *cooS* sequences of the non-acetogen *Carboxydibrachium pacificum* (NW_002243418) and the acetogen *C. difficile* (AM180355). The PCR product yielded a signal of approximately 1,800 base pairs. These primers targeted *T. kivui*, *C. drakei*, *C. magnum*, *T. glycolicus*, and *A. woodii* but yielded no signal from genomic DNA of *Moorella* and *Sporomusa* species. The *cooS* sequences of *A. woodii*, *T. kivui*, *C. magnum, C. drakei*, and *T. glycolicus* were sequenced (Table 20) and used for further design of primers. Subsequently, the primers cooS103f-I, cooS103f-II, cooS634r, cooS896r-I, and cooS896r-II were designed and evaluated (Table 21, Table 22, Table 23).

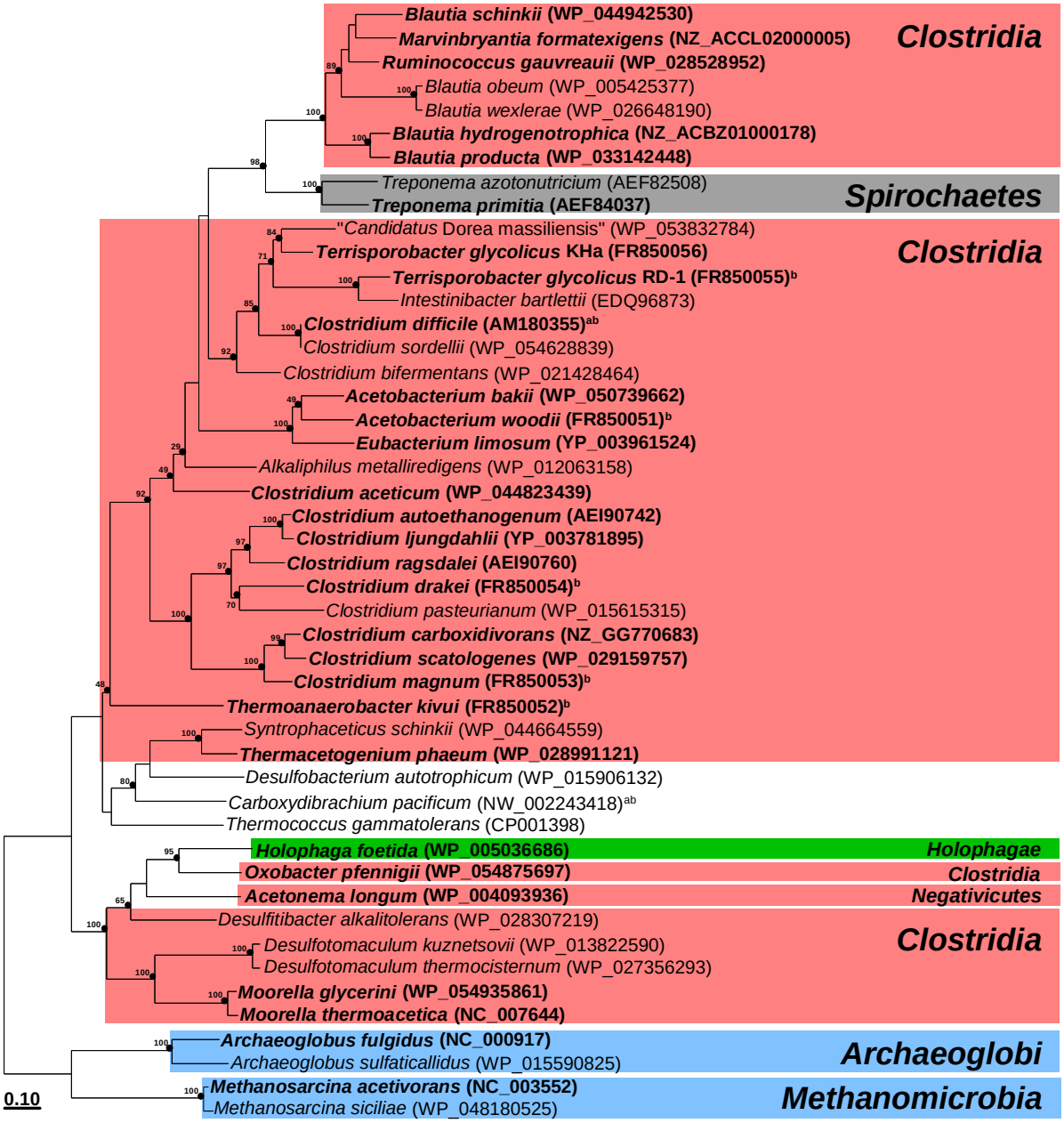


Figure 16: Phylogenic maximum parsimony tree of *in silico*-translated amino acid sequences encoded by *cooS* retrieved from acetogens (bold) and next cultured non-acetogens.

Sequences displayed in phylogenetic tree were obtained between 2009 and 2016. Accession numbers are indicated. Sequences correspond to residues 36 to 317 of the *cooS*-encoded amino acid sequence of *A. fulgidus* (NC_000917). The phylogenetic tree was calculated using the maximum parsimony method. Filled circles indicate congruent nodes in the neighbor joining tree, and the maximum likelihood tree. Bootstrap values derived from the maximum parsimony tree (1,000 resamplings) and are only displayed at nodes congruent in all three trees. The bar indicates a 0.1 change per amino acid. Phyla were displayed color coded: red, *Firmicutes*; grey, *Spirochaetae*; green, *Acidobacteria*; blue, *Euryarchaeota*. Sequences that were used for primer design in 2009 were indicated: [a], cooS805f/cooS2623rw; [b], all other primers.

The primers cooS103f-I, cooS103f-II, and cooS634r showed a signal of approximately 550 base pairs with genomic DNA from *T. kivui*. The primers cooS103f-I, cooS103f-II, cooS896r-I, and cooS896r-II showed a signal of approximately 800 base pairs with genomic DNA from *T. kivui*. With the latter two primer assays, genomic DNA of the non-acetogen *Methanosarcina mazei* did not yield a PCR signal and genomic DNA of other acetogens beside *T. kivui* were not tested for PCR signals.

Unfortunately, all *fhs* and *cooS* primer assays showed no PCR signal with DNA extracts from environmental samples (such as mire 'soils', roots or gut contents of earthworms). Since the design of the primers, new *fhs* and *cooS* sequences of acetogens became available (Figure 16) and primers that would target a broader range of acetogens could likely be designed and might deliver PCR signals from environmental samples of interest.

5.1.2 Phylogenic correlation of 16S rRNA gene sequences to *mcrA* and bacterial *fhs* sequences

McrA encodes the alpha-subunit of the methyl-CoM reductase of methanogens (Lehmacher and Klenk 1994, Pihl *et al.* 1994, Thauer 1998) whereas *fhs* encodes the formyl-tetrahydrofolate synthetase of acetogens and other bacteria (Leaphart and Lovell 2001, Müller *et al.* 2013). *mcrA* and *fhs* were used in this study to identify methanogens and acetogens, respectively. Threshold values are helpful to cluster obtained sequences into phylotypes. For example, a similarity threshold of 97 % can be used to cluster 16S rRNA gene sequences into species-level phylotypes (Yarza *et al.* 2008). Corresponding threshold values were calculated for *mcrA* and *fhs*.

Sequences of both genes were *in silico* translated into amino acids and plotted together with the corresponding 16S rRNA gene sequences of the same microorganisms (Figure 17). A threshold of 97 % and 87.5 % 16S rRNA gene sequence similarity (Yarza *et al.* 2008) was used to identify species- and family-level phylotypes, respectively. Based on the plot of Figure 17 corresponding thresholds were determined for *mcrA*-encoded and *fhs*-encoded amino acid sequences. A species- and family-level threshold for (a) *mcrA*-encoded amino acid sequences

of 85.7 % and 75.4 %, and (b) bacterial *fhs*-encoded amino acid sequences of 76.4 % and 50.0 % were obtained for species- and family-level phylotypes, respectively. Those threshold values were conservative and allowed an estimation of the minimal number of species- and family-level phylotypes in this dissertation.

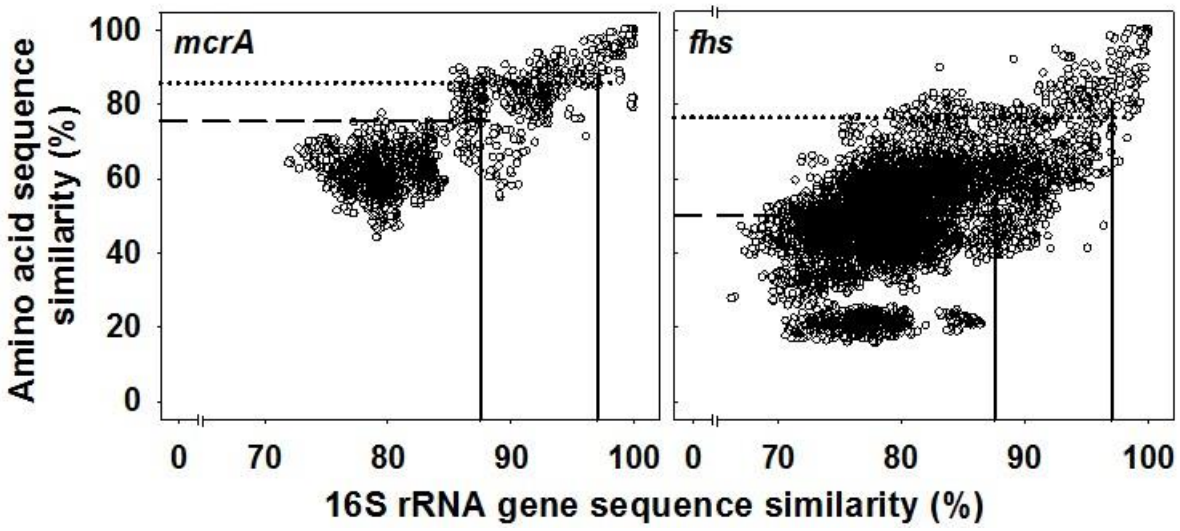


Figure 17: Phylogenic correlation plots of 16S rRNA gene sequence similarities and amino acid sequence similarities of *mcrA* and *fhs*.

Seventy-nine *mcrA* and 235 *fhs* sequences are plotted. Sequences that were used can be found in Table 46 and Table 47. The vertical solid lines intersecting the horizontal axes at 97 % and 87.5 % 16S rRNA gene sequence similarity identify thresholds for species- and family-level phylotypes, respectively (Yarza *et al.* 2008). The horizontal dotted and dashed lines intersecting the left vertical axes represent the 90 % quantile of pair wise comparisons of *mcrA*- or *fhs*-encoded amino acid sequence similarity and the 16S rRNA gene sequence similarity. Figure was modified from Hunger *et al.* (2011a).

5.2 Similarities and dissimilarities of anaerobic processes and associated taxa in contrasting mire 'soils'

5.2.1 Abiotic characteristics

The four mire 'soils' varied significantly in pH ($p < 0.001$), water content ($p < 0.002$), concentration of total carbon ($p < 0.001$), concentration of total nitrogen ($p < 0.002$), concentration of PO_4^{3-} ($p < 0.04$), and had different vegetation's (Table 24). Concentrations of Cl^-, NO_3^-, and SO_4^{2-} were similar ($p > 0.06$) in the contrasting mire 'soils', and NPOC increased with decreasing pore water pH. Except of mire 1, all other mire 'soils' contained peat. The concentration of total carbon increased and the concentration of total nitrogen decreased with

increasing water content. Based on C/N ratio and pH (Joosten 2001), the 'soil' of mire 1 was eutrophic pH-neutral, the 'soil' of mire 2 was mesotrophic acidic, and the 'soils' of mires 3 and 4 were oligotrophic acidic.

Table 24: Characteristics of contrasting mires in Germany.[a]

	Mire 1	Mire 2	Mire 3	Mire 4
Type of mire	Fen	Fen	Bog	Bog
Presence of peat	No	Yes	Yes	Yes
pH[b]	7.6 ± 0.4	4.3 ± 0.1	4.9 ± 0.9	3.9 ± 0.1
Water content (%)	81 ± 4	87 ± 6	93 ± 6	92 ± 2
Total C (g kg^{-1})[c]	259 ± 70	380 ± 31	459 ± 6	482 ± 13
Total N (g kg^{-1})[c]	20 ± 4	17 ± 4	10 ± 1	9 ± 1
C/N ratio	13	22	46	53
NPOC (mg L^{-1})[bd]	79 ± 32	115 ± 31	105 ± 36	171 ± 21
Cl^- (mg L^{-1})[b]	7.2 ± 4.7	2.1 ± 0.9	3.2 ± 1.9	5.7 ± 1.1
NO_3^- (mg L^{-1})[b]	-	0.2 ± 0.1	1.0 ± 0.4	0.8 ± 0.4
PO_4^{3-} (mg L^{-1})[b]	-	0.3 ± 0.1	-	4.2 ± 2.2
SO_4^{2-} (mg L^{-1})[b]	7.2 ± 1.6	18 ± 5	13 ± 3	8.6 ± 6.2
Location	Oberpfalz	Fichtelgebirge	Oberpfalz	Erzgebirge

[a] Table was modified from Hunger *et al.* (2015). Legend: -, not detected.
[b] Of pore water.
[c] Of soil dry weight.
[d] Non-purgeable organic carbon (NPOC) in filtered liquid samples.

5.2.2 Product profiles of anoxic unsupplemented 'soil' slurries

CO_2 and CH_4 accumulated and traces of acetate, lactate, and formate were detected in all unsupplemented slurries (Figure 18, data for lactate and formate not shown), suggesting that methanogenesis, fermentation, and/or anaerobic respiration occurred at the expense of endogenous substrates.

5.2.3 Effect of supplemental glucose on product profiles of anoxic 'soil' slurries

Glucose consumption stimulated the production of various fermentation products and CH_4 in all anoxic slurries (Figure 18, Table 25). Most abundant products were butyrate, acetate, and CO_2.

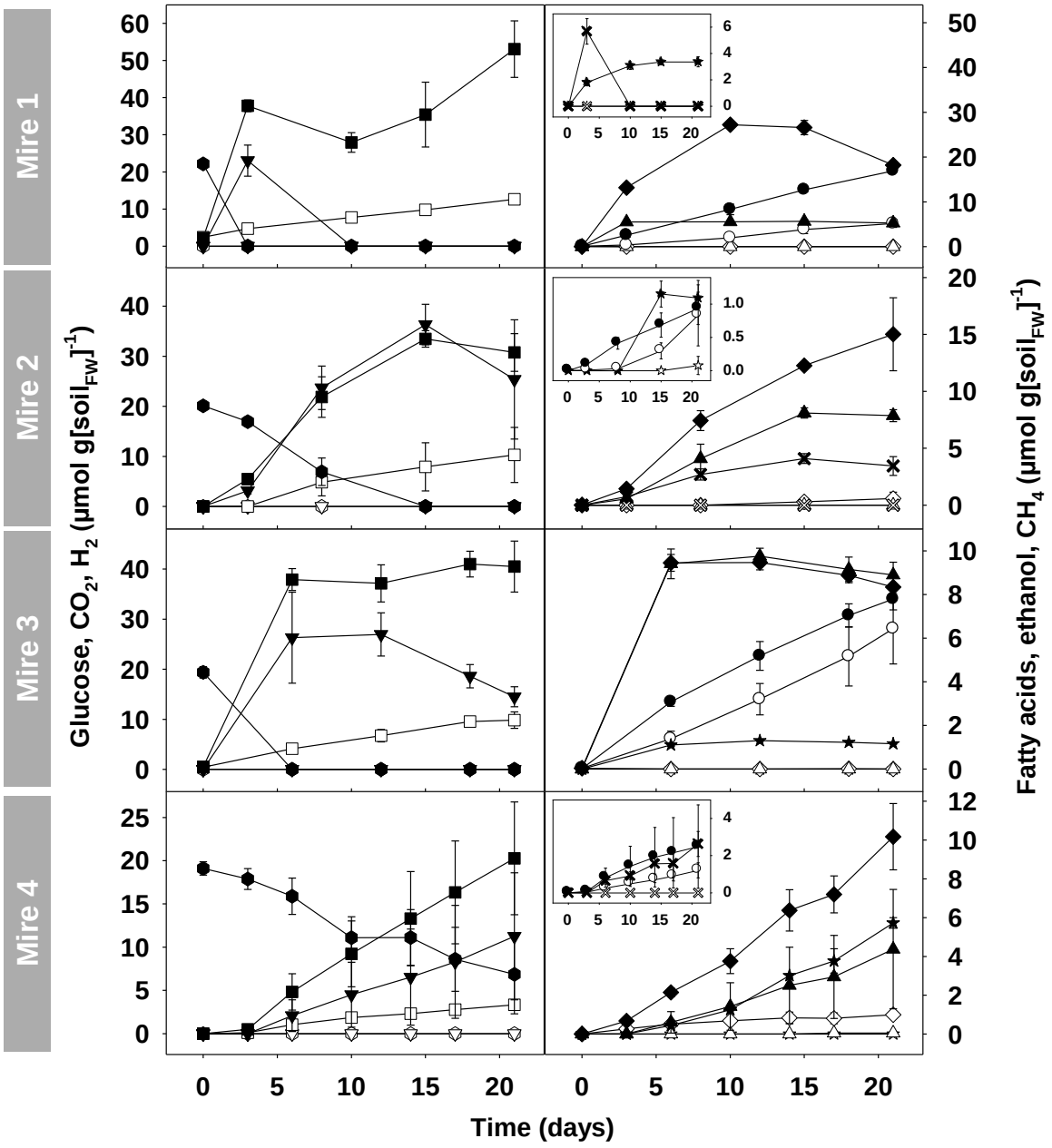


Figure 18: Effect of supplemental glucose on the production of organic acids and gases in anoxic 'soil' slurries derived from contrasting mires.

The incubation temperature was 15 °C. Symbols: open symbols, unsupplemented controls; closed symbols, glucose-supplemented slurries; hexagon, glucose; cross, ethanol; diamond, acetate; pyramid, butyrate; star, propionate; square, CO_2; upside-down triangle, H_2; circle, CH_4. Glucose was supplemented once at the beginning of incubation. Values are the means of triplicate slurries. Error bars indicate the standard deviation. Figure was modified from Hunger *et al.* (2015).

Theoretical recovery of glucose-derived carbon and reductant in slurries from all mires indicated that 21-40 % of the reductant was recovered in butyrate, 16-24 % of the reductant was recovered in acetate, and 21-29 % of the carbon was recovered in CO_2 (Table 25). Up to 13 % and 15 % of reductant from glucose were theoretically recovered in ethanol and H_2,

respectively. Ethanol was produced during the degradation of glucose in slurries from mires 1, 2, and 4, but not in slurries from mire 3 (Figure 18). Accumulated H_2 was consumed after glucose was completely degraded. Acetate accumulated during the utilization of H_2 and CO_2 in slurries from mire 1. The consumption of CO_2 and the accumulation of acetate were not observed after depletion of H_2, indicating that acetogenesis was an ongoing process until H_2 was depleted. Propionate was a minor product of the degradation of glucose in slurries from mires 1-3 (3-5 % of glucose-derived reductant) but more abundant in slurries of mire 4 (18 % of glucose-derived reductant). Formate was detected periodically in trace amounts in all slurries, suggesting that varying amounts of formate might have been formed and utilized during the different incubations.

Table 25: Recovery of glucose-derived reductant and carbon in anoxic slurries of contrasting mire 'soils'.

	Carbon recovered from glucose (%)[a]				Reductant recovered from glucose (%)[a]			
	Mires				Mires			
Products	1	2	3	4	1	2	3	4
Acetate	20	20	16	24	20	20	16	24
Butyrate	17	27	32	22	21	34	40	27
Propionate	4	3	3	15	5	3	3	18
Ethanol	9	7	0	4	13	10	0	7
Formate	4	0	0	0	2	0	0	0
CO_2	25	21	29	23	n.a.	n.a.	n.a.	n.a.
CH_4	2	0	1	3	3	1	3	3
H_2	n.a.	n.a.	n.a.	n.a.	9	15	11	8
Total :	81	78	81	91	73	83	73	87

[a] Recovery was calculated based on process data at the beginning of incubation and the following time points that corresponded to either when glucose was fully consumed or the end of the incubation (see Figure 18): mire 1, three days incubation; mire 2, 15 days incubation; mire 3, six days incubation; mire 4, 21 days incubation. Values are rounded to nearest whole number. Abbreviation: n.a., not applicable.

5.2.4 Effect of supplemental H_2-CO_2 on product profiles of anoxic 'soil' slurries

Supplemental H_2-CO_2 stimulated the production of acetate and CH_4 in all anoxic slurries (Figure 19, Table 26). The consumption of CO_2 and H_2 was concomitant with the formation of

acetate (Figure 19), an activity indicative of H_2-dependent acetogenesis. Acetate was consumed as soon as H_2 was depleted.

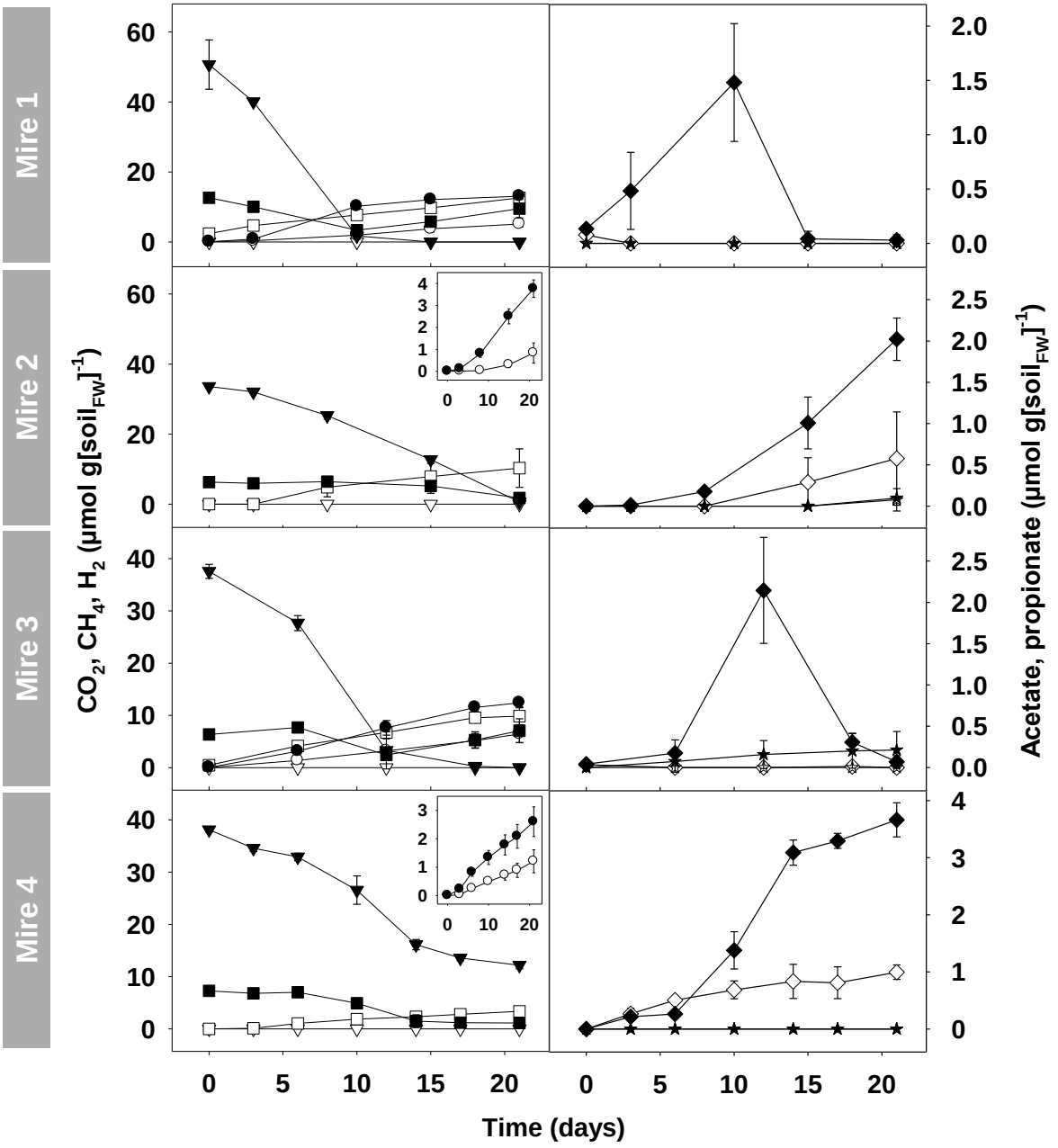


Figure 19: Effect of supplemental H_2-CO_2 on the production of acetate and CH_4 in anoxic 'soil' slurries derived from contrasting mires.

The incubation temperature was 15 °C. Symbols: open symbols, unsupplemented controls; closed symbols, H_2-CO_2-supplemented slurries; diamond, acetate; star, propionate; square, CO_2; upside-down triangle, H_2; circle, CH_4. H_2 and CO_2 were supplemented once at the beginning of incubation. Values are the means of triplicate slurries. Error bars indicate the standard deviation. Figure was modified from Hunger *et al.* (2015).

CH_4 accumulated as long as H_2 and/or acetate were present, which is indicative of hydrogenotrophic and aceticlastic methanogenesis, respectively. The apparent production of

CH_4 from H_2-CO_2 occurred before the apparent H_2-CO_2-dependent production of acetate in slurries from mires 2-4, whereas the apparent production of acetate from H_2-CO_2 occurred before the apparent H_2-CO_2-dependent production of CH_4 in slurries of mire 1. These contrasting patterns reflected the variability of competing processes (i.e. acetogenesis and methanogenesis) for H_2-derived reductant.

Table 26: Recovery of reductant from H_2 and recovery of carbon from CO_2 in anoxic H_2-CO_2-supplemented slurries of contrasting mire 'soils'.

	Carbon recovered from CO_2 (%)[a]				Reductant recovered from H_2 (%)[a]			
	Mires				Mires			
Products	1	2	3	4	1	2	3	4
Acetate	20	20	42	58	12	18	25	43
CH_4	57	20	43	15	67	36	52	22
Total :	77	40	85	73	79	54	77	65

[a] Recovery was calculated based on process data at the beginning of incubation and the following time points that corresponded to the highest acetate concentration (see Figure 19): mire 1, 10 days incubation; mire 2, 21 days incubation; mire 3, 12 days incubation; mire 4, 21 days incubation. Values are rounded to nearest whole number.

The recovery of reductant in CH_4 and acetate varied in 'soil' slurries from the contrasting mires (Table 26). 22-67 % of H_2-derived reductant was recovered in CH_4 and 12-43 % of H_2-derived reductant was recovered in acetate. Recovery calculations for 'soil' slurries from mires 1 and 3 were done in the middle of the incubation at a time point when acetate was detected transiently and acetate concentrations were the highest (Figure 19). It is unresolved how much of the H_2-CO_2-derived acetate was already converted to CH_4 at those time points and thus might shift the recovery of H_2-CO_2-derived carbon and reductant from acetate towards CH_4.

5.2.5 Effect of supplemental acetate on product profiles of anoxic 'soil' slurries

Supplemental acetate stimulated the production of CH_4 in all slurries (Figure 20). H_2 was transiently produced during the degradation of acetate in slurries from mires 1 and 3.

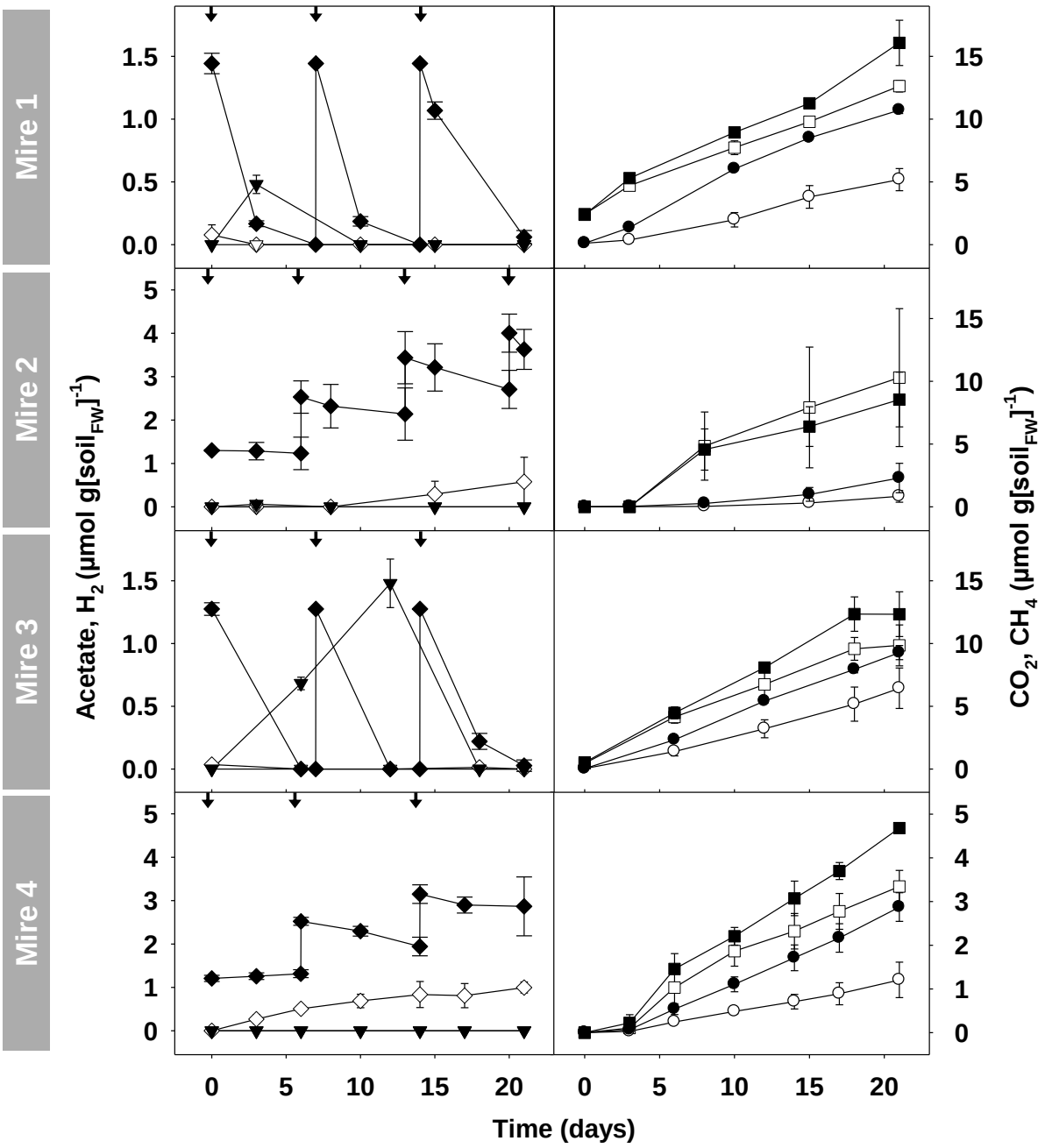


Figure 20: Effect of supplemental acetate on the production of gases in anoxic 'soil' slurries derived from contrasting mires.

The incubation temperature was 15 °C. Symbols: open symbols, unsupplemented controls; closed symbols, acetate-supplemented slurries; diamond, acetate; square, CO_2; upside-down triangle, H_2; circle, CH_4. Acetate was supplemented repeatedly during the incubation and time points of supplementation were indicated with arrows. Values are the means of triplicate slurries. Error bars indicate the standard deviation. Figure was modified from Hunger *et al.* (2015).

Aceticlastic methanogenesis has a theoretical stoichiometry of produced CO_2 to CH_4 of 1:1 (Zinder 1994). 'Soil' slurries from mire 1, 3, and 4 showed a stoichiometry of produced

CO_2 to CH_4 of 1:1.6, 1:1.2, and 1:1.2, respectively (Table 27), indicating that CH_4 was not exclusively derived from supplemental acetate but also from endogenous substrates. In this regard, the theoretical recovery of reductant above 100 % in slurries of mires 1 and 2 also indicated that supplemental acetate enhanced the utilization of endogenous substrates.

Table 27: Recovery of reductant and carbon from acetate in anoxic acetate-supplemented slurries of contrasting mire 'soils'.

	Carbon recovered from acetate (%)[a]				Reductant recovered from acetate (%)[a]			
	Mires				Mires			
Products	1	2	3	4	1	2	3	4
CO_2	43	0	33	33	n.a.	n.a.	n.a.	n.a.
CH_4	68	69	39	41	137	138	77	80
Total :	111	69	72	74	137	138	77	80

[a] Recovery was calculated based on process data at the beginning of incubation and day 21 (see Figure 20). Values are rounded to nearest whole number. Abbreviation: n.a., not applicable.

5.2.6 Bioenergetics of H_2-dependent methanogenesis and acetogenesis

The estimated Gibbs free energy of H_2-dependent methanogenesis (i.e. -32 to -100 kJ mol^{-1}) and acetogenesis (i.e. -21 to -108 kJ mol^{-1}) indicated that those processes were thermodynamically feasible under the experimental conditions (Figure 21). The estimated Gibbs free energy of acetogenesis was 8-14 kJ mol^{-1} more negative than of methanogenesis in slurries of mire 1. In contrast, the estimated Gibbs free energy of methanogenesis was 19-43 kJ mol^{-1}, 23-32 kJ mol^{-1}, and 23-30 kJ mol^{-1} more negative than that of acetogenesis in slurries of mires 2, 3, and 4, respectively.

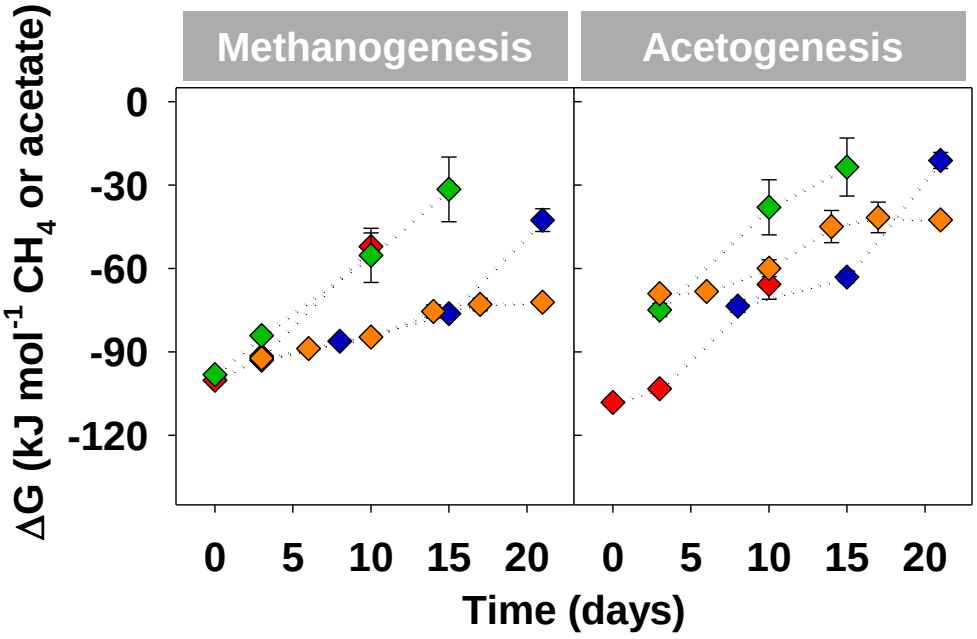


Figure 21: Estimated changes of the Gibbs free energy (ΔG) in H_2-CO_2-supplemented slurries for H_2-CO_2-dependent methanogenesis and H_2-CO_2-dependent acetogenesis.

Process data are plotted in Figure 19. Color code for derivation of 'soil' slurries: red, mire 1; blue, mire 2; green; mire 3; orange, mire 4. Values are the means of triplicate slurries. Error bars indicate the standard deviation. Figure was modified from Hunger *et al.* (2015).

5.2.7 Diversity of *mcrA* phylotypes

In total, 931 *mcrA* sequences clustered into 20 species-level and 10 family-level *mcrA* phylotypes (Table 28, Figure 24); including *Methanobacteriaceae*, *Methanocellaceae*, *Methanoregulaceae*, "*Methanosaetaceae*", *Methanosarcinaceae*, and five family-level phylotypes without any cultured isolates (Figure 23, Figure 24). Rarefaction curves and coverage indicated that sampling was sufficient for species-level clustering of *mcrA* phylotypes (Figure 22, Table 28). The diversity of species-level *mcrA* phylotypes decreased from mire 1 to mire 2, to mire 3, and mire 4. In this regard, water content and the C/N ratio increased (Table 24) with decreasing diversity of *mcrA* phylotypes, suggesting a correlation between the diversity of *mcrA* phylotypes, water content and C/N ratio.

Table 28: Coverage of clone libraries, number of *mcrA* sequences, and number of species-level *mcrA* phylotypes obtained from contrasting mire 'soils' and slurries of anoxic incubations.

Mires	Clone libraries		No. of sequences	No. of phylotypes	Coverage
1	Mire 'soil'		41	8	95
	Unsupplemented control[a]		47	8	94
	Glucose supplementation[a]		48	11	90
	Acetate supplementation[a]		50	10	92
	H_2-CO_2 supplementation[a]		46	8	98
		Total:	232	14	>99
2	Mire 'soil'		46	8	93
	Unsupplemented control[a]		45	10	96
	Glucose supplementation[a]		47	9	98
	Acetate supplementation[a]		48	10	98
	H_2-CO_2 supplementation[a]		40	6	98
		Total:	226	12	>99
3	Mire 'soil'		48	8	98
	Unsupplemented control[a]		46	6	>99
	Glucose supplementation[a]		48	7	>99
	Acetate supplementation[a]		48	5	>99
	H_2-CO_2 supplementation[a]		48	7	98
		Total:	238	8	>99
4	Mire 'soil'		46	4	98
	Unsupplemented control[a]		47	5	94
	Glucose supplementation[a]		53	5	98
	Acetate supplementation[a]		45	5	96
	H_2-CO_2 supplementation[a]		44	4	98
		Total:	235	8	>99

[a] Sequences were obtained from 'soil' slurries after 21 days of anoxic incubation. Process data can be found in Figure 18, Figure 19, and Figure 20.

Methanoregulaceae was the most abundant taxon in all four mire 'soils' before incubation (Figure 23). "*Methanosaetaceae*" and *Methanocellaceae* were the second and third most abundant taxa in mire 'soil' 1, respectively. *Methanosarcinaceae* and "*Methano-saetaceae*" were the second and third most abundant taxa in mire 'soil' 2, respectively.

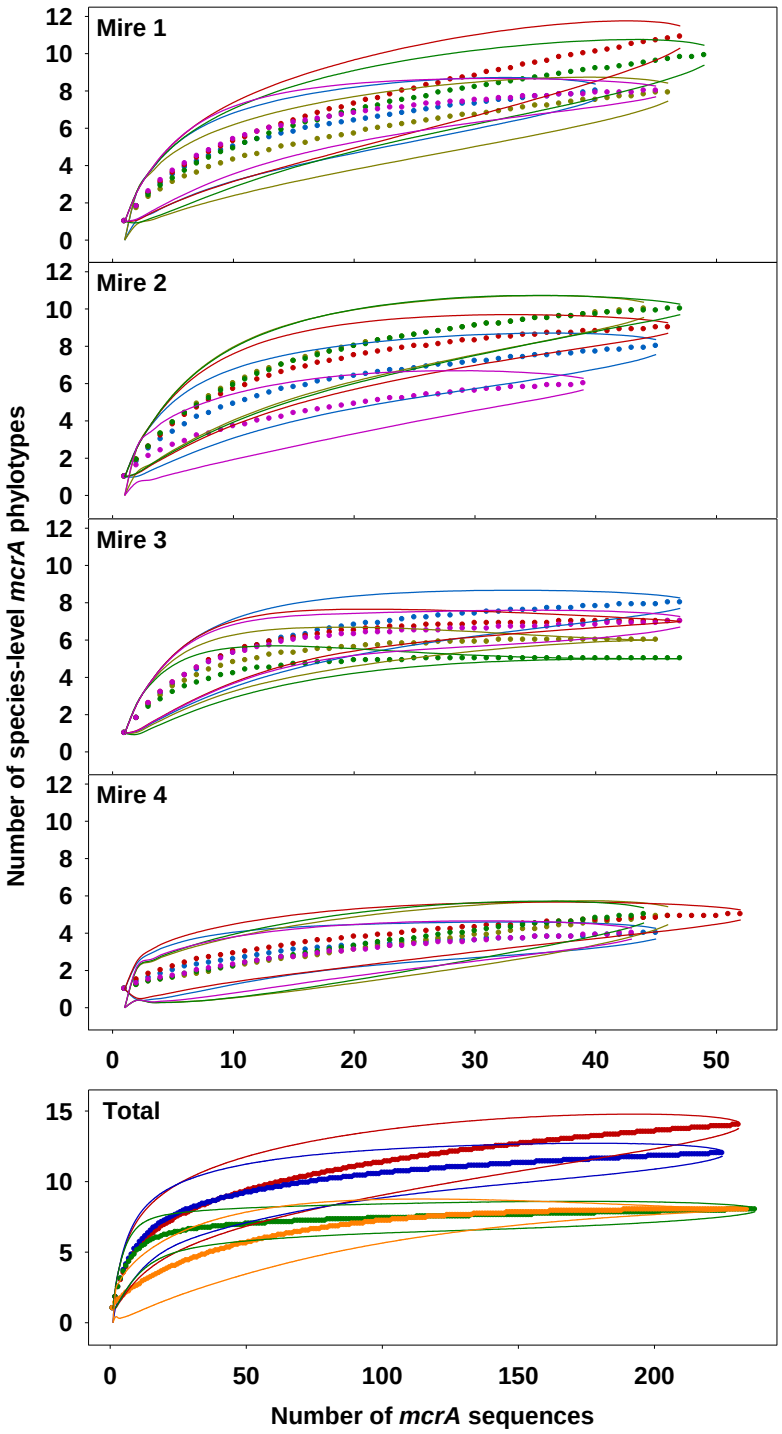


Figure 22: Rarefaction analysis of species-level *mcrA* phylotypes obtained from 'soils' and 'soil' slurries of contrasting mires.

Sequences were obtained from 'soils' before incubation or from 'soil' slurries after 21 days of incubation. Process data can be found in Figure 18, Figure 19, and Figure 20. *mcrA* sequences were *in silico* translated into amino acids and based on an 85.7 % similarity cut-off clustered into species-level phylotypes (5.1.2). A 95 % confidence interval is shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Color code for plots of mires: blue, mire 'soil' before incubation; light green, unsupplemented control; red, glucose-supplemented slurries; dark green, acetate-supplemented slurries; pink, H_2-CO_2-supplemented slurries. Color code for plot of total number of sequences per mire: red, mire 1; blue, mire 2; green, mire 3; orange, mire 4.

In mire 'soil' 3, *Methanosarcinaceae* and family-level phylotype 1 were the second and third most abundant taxa, respectively. "*Methanosaetaceae*" and *Methanosarcinaceae* were the second and third most abundant taxa in mire 'soil' 4, respectively. These results indicate, that (a) in general *mcrA*-associated communities varied between contrasting mire 'soils and (b) *Methanoregulaceae* was the most abundant taxon, indicating that *Methanoregulaceae*-related taxa might play a major role in mire 'soils'.

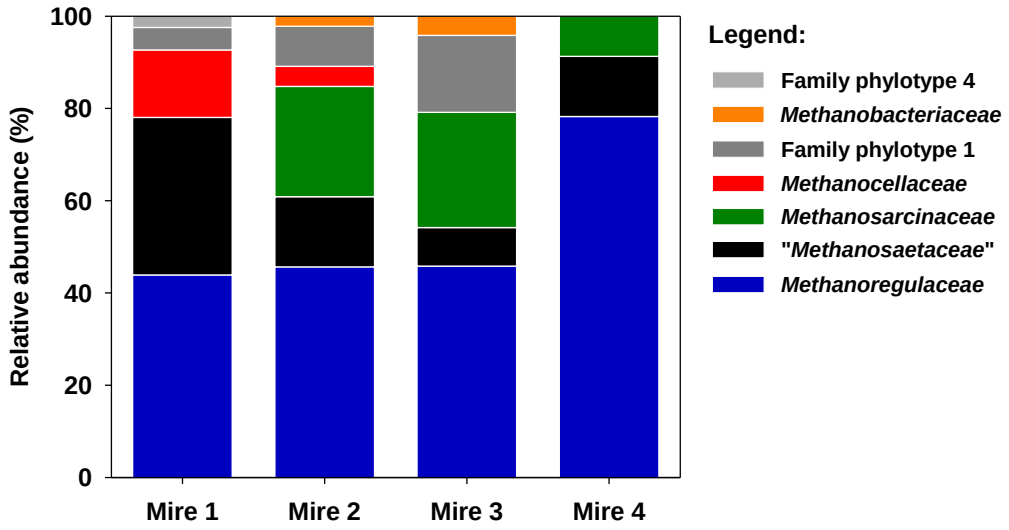


Figure 23: Relative abundances of family-level *mcrA* phylotypes from contrasting mire 'soils'.

Samples were taken from mire 'soils' prior to incubation. *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net).

'Soil' slurries from mires 1, 2, 3, and 4 showed similar diversities of species-level *mcrA* phylotypes before and after incubation (Figure 22). Some species-level phylotypes had a relative abundance of 9-35 % in the mire 'soil' and decreased in abundance during incubation of the unsupplemented 'soil' slurries (e.g., PLT2 [*Methanoregulaceae*] and PLT12 [*Methanocellaceae*] in slurries of mire 1, PLT1 [*Methanoregulaceae*] and PLT14 [*Methanosarcinaceae*] in slurries of mire 2, PLT4 [*Methanoregulaceae*] in slurries of mire 3, and PLT14 and PLT16 ["*Methanosaetaceae*"] in slurries of mire 4) (Figure 24), indicating that the experimental conditions did not favor the growth of associated methanogens. Other species-level phylotypes increased in relative abundance in 'soil' slurries after supplementation with substrate stronger than in the unsupplemented controls (e.g., PLT1 and PLT2 in slurries of mire 2 after supplementation of H_2-CO_2, PLT7 [family-level phylotype 1] in slurries of mire 1

after supplementation of H_2-CO_2, and PLT14 in slurries of mires 1, 3, and 4 after supplementation of either glucose or acetate), indicating that associated methanogens were stimulated by H_2-CO_2, acetate, and/or glucose-derived fermentation products under the experimental conditions.

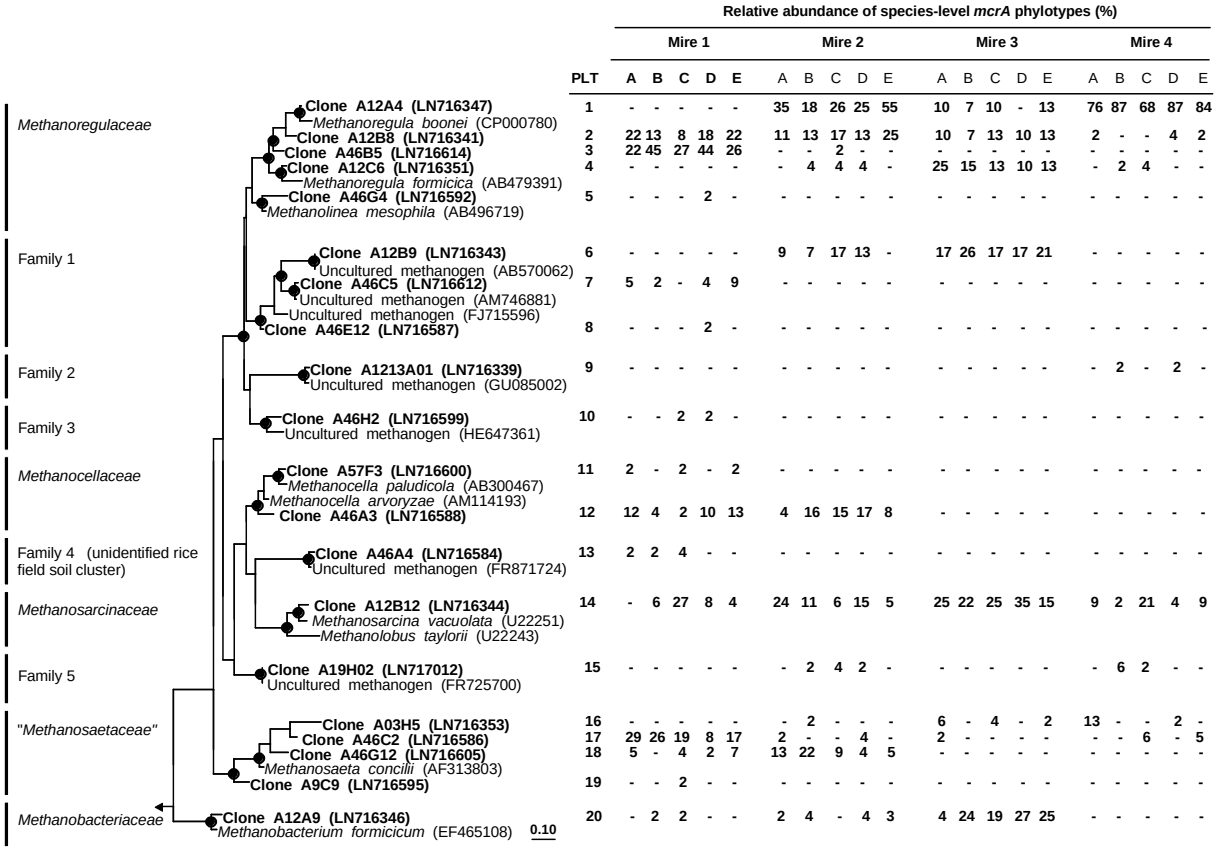


Figure 24: Phylogenic maximum likelihood tree of (a) representative *mcrA*-encoded amino acid sequences retrieved from contrasting mire 'soils' and from anoxic 'soil' slurries, and (b) reference sequences.

Sequences were obtained from mire 'soils' and from 'soil' slurries after 21 days of anoxic incubation (Figure 18, Figure 19, Figure 20). *mcrA* sequences were *in silico* translated into amino acids and based on an 85.7% similarity cut-off clustered into species-level phylotypes (5.1.2). Some species-level phylotypes were less than 75.4 % similar to *mcrA*-encoded amino acid sequences of cultured isolates and were clustered into family-level phylotypes 1-5 (5.1.2). *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (www.bacterio.net). Accession numbers are indicated in brackets. Sequences correspond to residues 339-470 of the *mcrA*-encoded amino acid sequence and 1017-1410 of the *mcrA* sequence of *Methanopyrus kandleri* (AE009439). Filled dots at nodes indicate the confirmation of tree topology by six calculations with the same data set whereby nucleic acid and corresponding amino acid sequences were used with maximum likelihood, neighbor-joining and maximum parsimony algorithms. *M. kandleri* (AE009439) was used as outgroup. Bar indicates a 0.1 estimated change per amino acid. Legend: A, mire 'soil'; B, unsupplemented control; C, glucose-supplemented slurries; D, acetate-supplemented slurries; E, H_2-CO_2-supplemented slurries; PLT, phylotype; -, not detected. Values are rounded to nearest whole number. Figure was modified from Hunger *et al.* (2015).

Species-level phylotypes with a relative abundance of 24 % or more had a total relative abundance of 29 %, 59 %, 50 %, and 76 % in 'soils' of mire 1 (pH 7.6), mire 2 (pH 4.3), mire 3 (pH 4.9), and mire 4 (pH 3.9), respectively (Figure 24), suggesting that acidic conditions restricted the relative number of dominant methanogenic phylotypes.

5.2.8 Diversity of bacterial 16S rRNA gene phylotypes

In total, 870 bacterial 16S rRNA gene sequences were obtained that clustered into 86 family-level and 13 phyla-level phylotypes (Table 29). Rarefaction curves and coverage indicated that sampling was not sufficient for family-level clustering of 16S rRNA gene phylotypes for single supplementations for mire 'soils' 1 and 2 but was sufficient for mire 'soils' 3 and 4 and for analysis of total detected phylotypes per mire 'soil' (Figure 22, Table 29). The diversity of family-level 16S rRNA gene phylotypes decreased from mire 1 to mire 2, mire 3, and mire 4 (Table 29, Figure 25). In this regard, water content and the C/N ratio increased (Table 24) with decreasing diversity of 16S rRNA gene phylotypes, suggesting a correlation between the diversity of 16S rRNA gene phylotypes, water content and C/N ratio.

The diversity of 16S rRNA gene phylotypes was similar within a single mire 'soil' and little affected by supplementation and incubation (Table 29, Figure 25). *Acidobacteria* and *Proteobacteria* were the most abundant phyla in mire 'soils', and the relative abundance of *Acidobacteria* (especially *Acidobacteriaceae*, Figure 26, Table 30, Table 48) increased with decreasing mire pore water pH (Table 24). *Actinobacteria* was the third most abundant taxon in mire 'soils' 1 and 3 but less abundant in 'soils' of mire 2 and 4. Besides the high relative abundance of *Acidobacteria* and *Proteobacteria*, 'soils' of contrasting mires varied in their bacterial community composition. Some family-level 16S rRNA gene phylotypes within the *Acidobacteria* with a relative abundance in the mire 'soil' of over 10 % decreased in relative abundance during the incubation period (e.g., phylotype 3 in mire 1, phylotype 5 in mire 2, and phylotype 1 in mire 4) (Table 30), indicating that the experimental conditions did not favor affiliated microorganisms, which were thus overgrown by other microorganisms.

Table 29: Coverage of clone libraries, number of 16S rRNA gene sequences, and number of family-level 16S rRNA gene phylotypes obtained from contrasting mire 'soils' and slurries of anoxic incubations.

Mires	Clone libraries	No. of sequences	No. of phylotypes	Coverage
1	Mire 'soil'	42	31	43
	Unsupplemented control[a]	39	25	64
	Glucose supplementation[a]	41	27	54
	Acetate supplementation[a]	53	32	64
	H_2-CO_2 supplementation[a]	34	21	59
	Total:	209	56	93
2	Mire 'soil'	48	18	85
	Unsupplemented control[a]	42	21	74
	Glucose supplementation[a]	48	18	75
	Acetate supplementation[a]	45	22	64
	H_2-CO_2 supplementation[a]	41	19	73
	Total:	224	50	92
3	Mire 'soil'	42	16	83
	Unsupplemented control[a]	47	11	94
	Glucose supplementation[a]	46	13	89
	Acetate supplementation[a]	42	15	81
	H_2-CO_2 supplementation[a]	46	13	89
	Total:	223	33	93
4	Mire 'soil'	41	8	95
	Unsupplemented control[a]	44	13	84
	Glucose supplementation[a]	43	14	88
	Acetate supplementation[a]	46	8	91
	H_2-CO_2 supplementation[a]	40	19	65
	Total:	214	30	94

[a] Sequences were obtained from 'soil' slurries after 21 days of anoxic incubation. Process data can be found in Figure 18, Figure 19, and Figure 20.

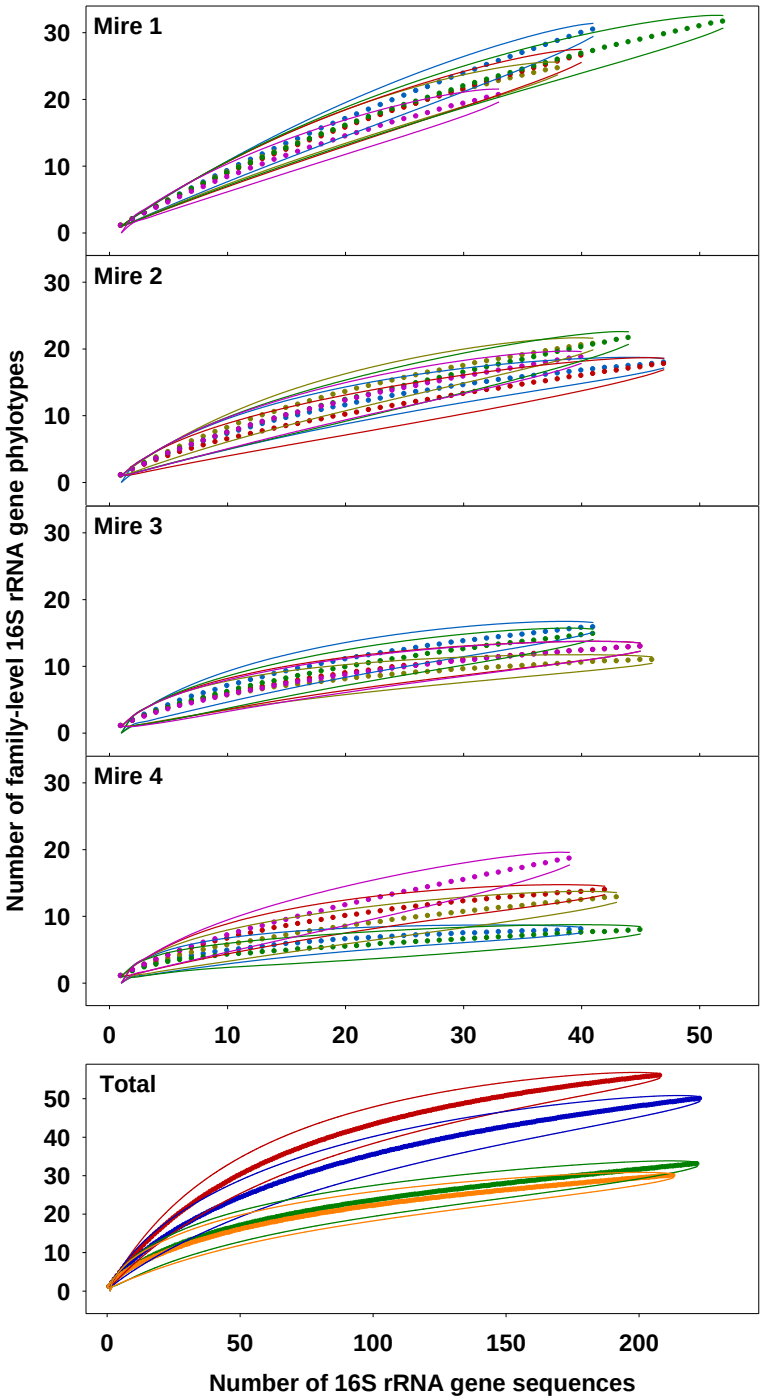


Figure 25: Rarefaction analysis of family-level 16S rRNA gene phylotypes obtained from 'soils' and 'soil' slurries of contrasting mires.

Sequences were obtained from 'soils' before incubation or from 'soil' slurries after 21 days of incubation. Process data can be found in Figure 18, Figure 19, and Figure 20. The 16S rRNA gene sequences were based on an 87.5 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). 95 % confidence intervals are shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Color code for plots of mires: blue, mire 'soil' before incubation; light green, unsupplemented control; red, glucose-supplemented slurries; dark green, acetate-supplemented slurries; pink, H_2-CO_2-supplemented slurries. Color code for plot of total number of sequences per mire 'soil': red, mire 1; blue, mire 2; green, mire 3; orange, mire 4.

Hyphomicrobiaceae, *Syntrophaceae*, and family-level phylotypes 28 were the most abundant taxa in overall gene libraries of mire 'soil' 1 (Table 30, Table 31), and collectively accounted for 12 % of the bacterial community before incubation (Table 48). *Anaerolineaceae*, and family-level phylotypes 3 and 41 were the most abundant family-level 16S rRNA gene phylotypes before incubation in mire 'soil' 1 (Table 30, Table 48). *Acidimicrobiaceae* increased in relative abundance due to incubation of unsupplemented 'soil' slurries from mire 1 (Table 30). Other taxa displayed a higher increase in relative abundance due to supplementation compared to unsupplemented controls during incubation in 'soil' slurries from mire 1, such as *Hyphomicrobiaceae* in glucose- and acetate-supplemented slurries, and family-level phylotypes 28 and 37 in H_2-CO_2-supplemented slurries.

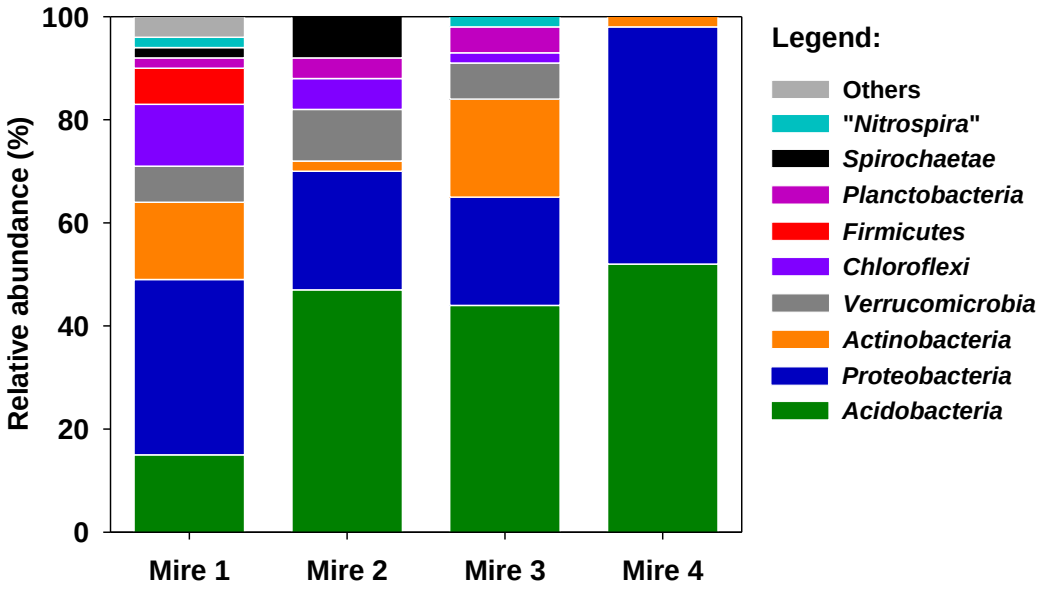


Figure 26: Relative abundance of phylum-level 16S rRNA gene phylotypes from contrasting mire 'soils'.

Sequences derived from mire 'soil' before incubation. Others include all sequences that could not be assigned to known phyla.

Acidobacteriaceae, *Planctomycetaceae*, and *Clostridiaceae* were the most abundant taxa in overall gene libraries of mire 'soil' 2 (Table 31), and collectively accounted for 29 % of the bacterial community before incubation (Table 30). *Acidobacteriaceae*, family-level phylotypes 1 and 5 were the most abundant family-level 16S rRNA gene phylotypes before incubation in mire 'soil' 2. Family-level phylotype 13 and *Planctomycetaceae* increased in

relative abundance due to incubation of unsupplemented 'soil' slurries from mire 2. Other taxa displayed higher increase in relative abundance due to supplementation compared to unsupplemented controls during incubation of 'soil' slurries from mire 2, such as *Clostridiaceae* in glucose-supplemented slurries and *Planctomycetaceae* in acetate-supplemented slurries.

Acidobacteriaceae, *Acidimicrobiaceae*, and family-level phylotype 1 were the most abundant taxa in overall gene libraries of mire 'soil' 3 (Table 31), and collectively accounted for 48 % of the bacterial community before incubation (Table 30). *Acidobacteriaceae* and *Acidimicrobiaceae* were also the most abundant family-level 16S rRNA gene phylotypes before incubation in mire 'soil' 3. *Acidobacteriaceae*, and family-level phylotypes 1 and 5 increased in relative abundance due to incubation of unsupplemented 'soil' slurries from mire 3. Other taxa displayed a higher increase in relative abundance due to supplementation compared to unsupplemented controls during incubation of 'soil' slurries from mire 3, such as *Acidobacteriaceae* in glucose- or acetate- or H_2-CO_2-supplemented slurries and *Planctomycetaceae* in glucose-supplemented slurries.

Acidobacteriaceae, *Methylocystaceae*, and *Acetobacteraceae* were the most abundant taxa in overall gene libraries of mire 'soil' 4 (Table 31), and collectively accounted for 73 % of the bacterial community before incubation (Table 30). *Acidobacteriaceae*, *Methylocystaceae*, *Acetobacteraceae*, and family-level phylotype 1 were the most abundant family-level 16S rRNA gene phylotypes before incubation in mire 'soil' 4. *Methylocystaceae* increased in relative abundance due to incubation of unsupplemented 'soil' slurries from mire 4. Other taxa displayed a higher increase in relative abundance due to supplementation compared to unsupplemented controls during incubation of 'soil' slurries from mire 4, such as *Clostridiaceae* and *Veillonellaceae* in glucose-supplemented slurries, *Veillonellaceae* in H_2-CO_2-supplementes slurries, and *Methylocystaceae* and *Acetobacteraceae* in acetate-supplemented slurries.

Table 30: Most abundant family-level phylotypes (i.e. phylotypes with a relative abundance of 10 % or higher), relative abundance of bacterial 16S rRNA gene sequences from mire 'soils', and from slurries at the end of the 21 day incubation.

	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
Taxonomic level (phylum, family)	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Acidobacteria																				
Acidobacteriaceae	-	-	-	4	-	25	14	15	20	27	29	34	39	40	43	41	32	23	24	28
Family-level phylotype 1[b]	-	3	-	2	6	8	-	2	-	5	7	19	4	2	13	10	2	-	-	8
Family-level phylotype 3[b]	10	3	2	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family-level phylotype 5[b]	-	-	2	2	-	13	-	-	-	7	5	11	11	-	9	-	-	-	-	-
Actinobacteria																				
Acidimicrobiaceae	2	10	-	6	3	-	7	2	9	2	12	11	9	10	4	2	-	-	-	5
Chloroflexi																				
Family-level phylotype 13[b]	-	-	-	-	-	2	12	-	-	5	-	-	-	-	-	-	-	-	-	-
Firmicutes																				
Clostridiaceae	5	3	5	-	-	-	2	31	2	-	-	-	2	-	-	-	2	21	-	-
Veillonellaceae	-	-	-	-	-	-	-	2	-	7	-	-	2	-	-	-	-	7	2	10
Planctobacteria																				
Planctomycetaceae	2	5	-	4	-	4	10	15	20	10	5	-	11	-	-	-	7	2	-	3
Proteobacteria																				
Hyphomicrobiaceae	2	5	15	13	6	6	5	6	2	-	-	-	2	7	4	-	-	-	-	-
Methylocystaceae	-	3	2	-	-	4	2	-	-	2	-	-	-	2	4	22	27	9	41	15
Acetobacteraceae	-	-	-	-	3	-	-	4	-	-	-	-	-	2	-	10	7	-	22	3
Family-level phylotype 28[b]	5	3	5	6	21	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-

[a] The 16S rRNA gene sequences were based on an 87.5 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). Values are rounded to nearest whole number. Legend: -, not detected; A, mire 'soil'; B, unsupplemented control; C, glucose-supplemented slurries; D, acetate-supplemented slurries; E, H_2-CO_2-supplemented slurries. Process data can be found in Figure 18, Figure 19, and Figure 20. Table was modified from Hunger *et al.* (2015).

[b] Sequences were considered to be family-level phylotypes without any cultured isolates if the 16S rRNA gene sequence was less than 87.5 % similar to the sequence of the closest related cultured species (Yashiro *et al.* 2011).

Table 31: Family-level phylotypes and relative abundances of total detected bacterial 16S rRNA gene sequences per mire 'soil' including sequences detected in slurries.

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]			
	Mire 1	Mire 2	Mire 3	Mire 4
Acidobacteria, Acidobacteria,				
Acidobacteriaceae	1.0	20.1	37.2	29.4
Family phylotype 1[b]	1.9	3.1	9.4	3.7
Acidobacteria, Holophagae,				
Holophagaceae	-	0.4	0.4	-
Acidobacteria, unknown class,				
Family phylotype 2[b]	0.5	-	-	-
Family phylotype 3[b]	3.8	-	-	-
Family phylotype 4[b]	3.3	-	-	-
Family phylotype 5[b]	1.0	4.0	7.2	-
Family phylotype 6[b]	-	0.4	0.4	0.5
Actinobacteria, Actinobacteria,				
Acidimicrobiaceae	4.3	4.0	9.0	1.4
Mycobacteriaceae	-	0.4	-	-
Thermomonosporaceae	0.5	1.3	3.6	0.5
Conexibacteraceae, Patulibacteraceae, Solirubrobacteraceae	2.9	1.8	3.1	0.9
Actinobacteria, unknown class,				
Family phylotype 7[b]	1.0	0.4	-	-
Family phylotype 8[b]	2.4	-	0.9	-
Family phylotype 9[b]	1.4	-	-	-
Family phylotype 10[b]	1.0	-	0.4	-
Armatimonadetes, Armatimonadia,				
Armatimonadaceae	-	-	-	0.9
Bacteroidetes, Bacteroidia,				
Family phylotype 11[b]	-	-	-	0.5
Bacteroidetes, Cytophagia,				
Cytophagaceae	1.9	-	-	-
Bacteroidetes, Sphingobacteriia,				
Chitinophagaceae	0.5	0.4	0.4	0.5
Family phylotype 12[b]	0.5	-	-	0.5

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]			
	Mire 1	Mire 2	Mire 3	Mire 4
Chlorobi, Ignavibacteria,				
Ignavibacteriaceae	-	0.4	-	-
Chloroflexi, Anaerolineae,				
Anaerolineaceae	3.8	1.3	0.4	-
Chloroflexi, Ktedonobacteria,				
Ktedonobacteraceae	-	0.9	-	-
Family phylotype 13[b]	-	3.6	-	-
Family phylotype 14[b]	-	0.4	-	-
Chloroflexi, unknown class,				
Family phylotype 15[b]	1.4	-	-	-
Family phylotype 16[b]	-	0.4	-	0.5
Family phylotype 17[b]	-	0.4	-	0.5
Family phylotype 18[b]	1.0	-	-	-
Family phylotype 19[b]	1.0	-	-	-
Family phylotype 20[b]	-	1.8	-	-
Cyanobacteria, unknown class,				
Family phylotype 21[b]	-	0.4	0.9	1.9
Firmicutes, Bacilli,				
Bacillaceae	0.5	-	-	-
Firmicutes, Clostridia,				
Clostridiaceae	2.4	7.6	0.4	4.7
Peptococcaceae	-	0.9	-	-
Ruminococcaceae	1.4	0.4	-	0.9
Family phylotype 22[b]	1.4	-	-	-
Firmicutes, Negativicutes,				
Veillonellaceae	-	1.8	0.4	3.7
"*Nitrospirae*", "*Nitrospira*",				
"*Nitrospiraceae*"	1.9	-	0.4	-
Planctobacteria, Planctomycea,				
Planctomycetaceae	2.4	11.6	3.1	2.3
Proteobacteria, Alphaproteobacteria,				
Caulobacteraceae	-	0.4	0.4	-
Beijerinckiaceae	0.5	1.8	1.8	1.4
Bradyrhizobiaceae	2.9	1.3	2.7	2.8
Hyphomicrobiaceae	8.6	4.0	2.7	-

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]			
	Mire 1	Mire 2	Mire 3	Mire 4
Methylocystaceae	1.0	1.8	1.3	23.4
Rhizobiaceae	0.5	-	-	-
Xanthobacteraceae	0.5	-	-	-
Methyloceanibacter-related phylotype	1.0	-	-	-
Acetobacteraceae	0.5	0.9	0.4	8.4
Rhodospirillaceae	2.4	-	-	0.5
Family phylotype 23[b]	-	-	-	0.5
Family phylotype 24[b]	1.0	-	0.4	2.8
Family phylotype 25[b]	-	2.2	2.7	1.9
Proteobacteria, Betaproteobacteria,				
Comamonadaceae	1.4	-	-	-
Oxalobacteraceae	-	0.9	0.4	-
Neisseriaceae	-	1.3	-	1.9
Rhodocyclaceae	-	0.4	-	-
Family phylotype 26[b]	-	0.4	-	-
Family phylotype 27[b]	2.4	-	-	-
Family phylotype 28[b]	7.2	0.4	-	0.5
Proteobacteria, Gammaproteobacteria,				
Coxiellaceae	-	-	-	0.5
Moraxellaceae	2.9	0.4	-	-
Family phylotype 29[b]	1.0	-	-	-
Proteobacteria, Deltaproteobacteria,				
Bdellovibrionaceae	-	-	0.4	-
Desulfobacteraceae	1.0	-	-	-
Geobacteraceae	0.5	0.4	-	-
Phaselicystidaceae, Polyangiaceae[d]	0.5	1.3	-	-
Syntrophaceae	4.8	0.9	0.4	-
Syntrophobacteraceae	-	0.9	-	-
Syntrophorhabdaceae	0.5	-	-	-
Family phylotype 30[b]	-	0.4	1.8	-
Family phylotype 31[b]	1.4	0.9	1.8	-
Family phylotype 32[b]	2.4	-	-	-
Family phylotype 33[b]	1.0	-	-	-

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]			
	Mire 1	Mire 2	Mire 3	Mire 4
Spirochaetae, Spirochaetes,				
Spirochaetaceae	0.5	2.2	-	-
Family phylotype 34[b]	-	1.3	-	-
Unclassified phylotypes[e]				
Xiphinematobacter-related,	1.0	-	-	-
Unknown phylotypes[e]				
Family phylotype 35[b]	1.4	-	-	-
Family phylotype 36[b]	-	0.4	-	-
Family phylotype 37[b]	2.4	-	-	-
Family phylotype 38[b]	0.5	-	-	-
Family phylotype 39[b]	0.5	-	-	-
Family phylotype 40[b]	-	0.9	0.4	-
Verrucomicrobia, Opitutae,				
Opitutaceae	-	1.3	1.8	0.5
Verrucomicrobia, unknown class*,*				
Family phylotype 41[b]	3.3	3.6	2.2	1.9

[a] The 16S rRNA gene sequences were based on an 87.5 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). Values are rounded to nearest decimal and thus might not sum up to 100 %. Legend: -, not detected. Table was modified from Hunger *et al.* (2015).
[b] Sequences were considered to be a family-level phylotype without any cultured isolate if the 16S rRNA gene sequence was less than 87.5 % similar to the sequence of the closest related cultured species (Yarza *et al.* 2008).
[c] Closest related cultured species: 94.5-88.8 % 16S rRNA gene sequence similarity to *Conexibacter arvalis* (AB597950), 92.9-87.2 % 16S rRNA gene sequence similarity to *Patulibacter americanus* (AJ871306), and 96.8-88.1 % 16S rRNA gene sequence similarity to *Solirubrobacter soli* (AB245334).
[d] Closest related cultured species: 88.5-86.5 % 16S rRNA gene sequence similarity to *Byssovorax cruenta* (AJ833647), and 90.2-87.8 % 16S rRNA gene sequence similarity to *Phaselicystis flava* (EU545827).
[e] Listed family-level phylotypes do not necessarily belong to the same phylum or class.

Collectively, approximately half of the detected family-level phylotypes (i.e., 41 out of 86 phylotypes, Table 31) were without any cultured isolates, indicating a high degree of unknown and potentially novel ecosystem functions. Family-level phylotypes with a relative abundance of 10 % or higher had a total relative abundance of 10 %, 38 %, 41 %, and 83 % in ‘soils’ before incubation of mire 1 (pH 7.6), mire 2 (pH 4.3), mire 3 (pH 4.9), and mire 4 (pH 3.9), respectively (Table 30), suggesting that acidic conditions restricted the relative number of dominant bacterial phylotypes.

5.2.9 Gene copy numbers and cultivable cell numbers

Copy numbers of 16S rRNA genes and *mcrA* did not vary significantly ($p > 0.35$) between mire 'soils', indicating that the abundance of bacteria and methanogens were relatively uniform in the contrasting mire soils (Figure 27 A). Gene copy numbers of 16S rRNA genes were significantly greater ($p < 0.04$) than gene copy numbers of *mcrA* (i.e., 0.1-28.8 x 10^9 16S rRNA genes g[$soil_{DW}$]$^{-1}$ vs. 0.5-111.6 x 10^7 *mcrA* g[$soil_{DW}$]$^{-1}$) (Figure 27 A) in each mire 'soil'. The average ratios of gene copy numbers of 16S rRNA genes to *mcrA* of the contrasting mires approximated 55, 40, 40, and 75 for 'soils' of mire 1, 2, 3, and 4, respectively. Although the cultivable numbers of microbes capable of aerobic growth appeared to be somewhat greater in some cases than the cultivable number of microbes capable of anaerobic growth (Figure 27 B), the quantities of microorganisms capable of aerobic and anaerobic growth were not significantly different in the contrasting mire 'soils' ($p > 0.15$).

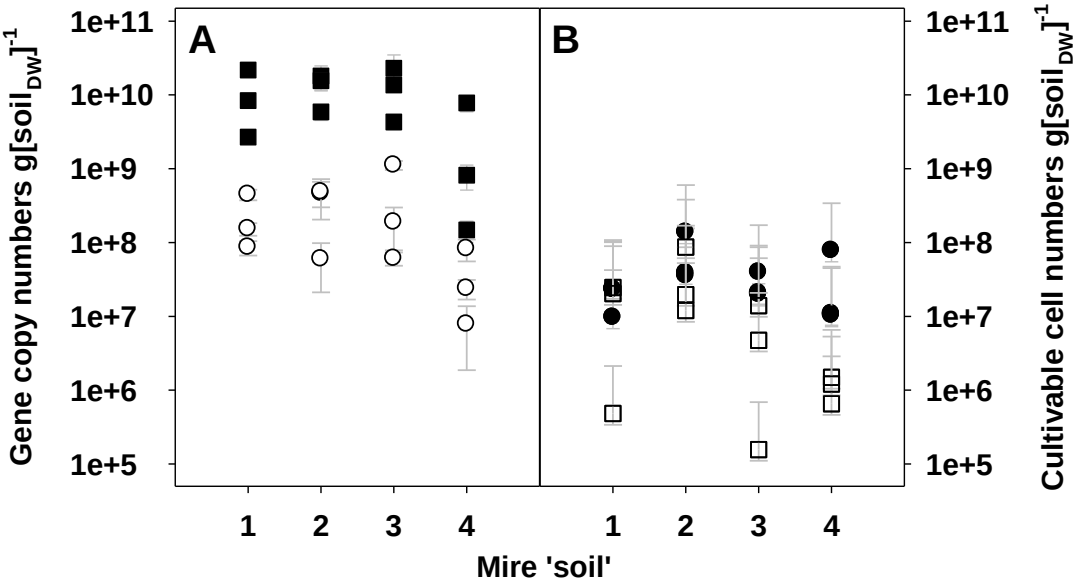


Figure 27: Quantities of gene copy numbers (A) and cultivable cell cumbers (B) in contrasting mire 'soils'.

Symbols that overlap appear as one symbol. Symbols: closed square, bacterial 16S rRNA gene copy numbers; open circle, *mcrA* gene copy numbers; closed circle, cultivable cell numbers under oxic conditions; open square, cultivable cell numbers under anoxic conditions. Values are the means of triplicate gene copy number analysis and duplicate MPN analysis. Error bars of gene copy numbers indicate the standard deviation. Error bars of cultivable cell numbers indicate the highest and lowest confidence interval (95 %). Figure was modified from Hunger *et al.* (2015).

5.3 Formate-dependent acetogenesis in moderately acidic mire 'soil'

5.3.1 Effect of supplemental formate on acetogenesis in anoxic 'soil' slurries

Alternative electron acceptors (i.e., approximately 103 µmol iron(III) and 15 µmol sulfate g[$soil_{DW}$]$^{-1}$) in anoxic 'soil' slurries from mire 2 were reduced during 15 days of anoxic pre-incubation prior to supplementation of [^{12}C]formate and [^{13}C]formate. Sulfate was not detected and iron(II) reached a stable final concentration at the end of the pre-incubation period. Nitrate was not detected during the pre-incubation period (detection limit was 0.13 µmol nitrate g[$soil_{DW}$]$^{-1}$). Approximately 2.5 µmol CH_4 g[$soil_{DW}$]$^{-1}$, 4 µmol acetate g[$soil_{DW}$]$^{-1}$, and 85 µmol CO_2 g[$soil_{DW}$]$^{-1}$ were produced at the end of the pre-incubation period.

Gases were removed prior to supplementation with formate. A total of approximately 25 µmol CH_4 g[$soil_{DW}$]$^{-1}$, 5 µmol acetate g[$soil_{DW}$]$^{-1}$, and 2 µmol propionate g[$soil_{DW}$]$^{-1}$ were produced in the subsequent 23 days after the preincubation in unsupplemented controls (Figure 28). Formate and H_2 remained below 1 µmol g[$soil_{DW}$]$^{-1}$ in unsupplemented controls.

Formate was supplemented each day and each formate pulse was essentially consumed within 24 hours (Figure 28). In total, approximately 280 µmol formate g[$soil_{DW}$]$^{-1}$ were supplemented within 23 days of anoxic incubation. In total, 63 µmol H_2 g[$soil_{DW}$]$^{-1}$, 43 µmol CH_4 g[$soil_{DW}$]$^{-1}$, 29 µmol acetate g[$soil_{DW}$]$^{-1}$, and 8 µmol propionate g[$soil_{DW}$]$^{-1}$ more were produced in formate-supplemented slurries compared to unsupplemented controls, indicating that formate stimulated the production of these compounds. Concentrations of gases never exceeded 165 µmol CO_2 g[$soil_{DW}$]$^{-1}$, 14 µmol CH_4 g[$soil_{DW}$]$^{-1}$, and 16 µmol H_2 g[$soil_{DW}$]$^{-1}$ due to exchanges of the gas phase. The apparent formate-dependent production of H_2 suggested that taxa harboring a FHL complex, an enzyme complex that converts formate to H_2 and CO_2 (Vignais and Billoud 2007, Trchounian and Sawers 2014), were active in formate-supplemented slurries.

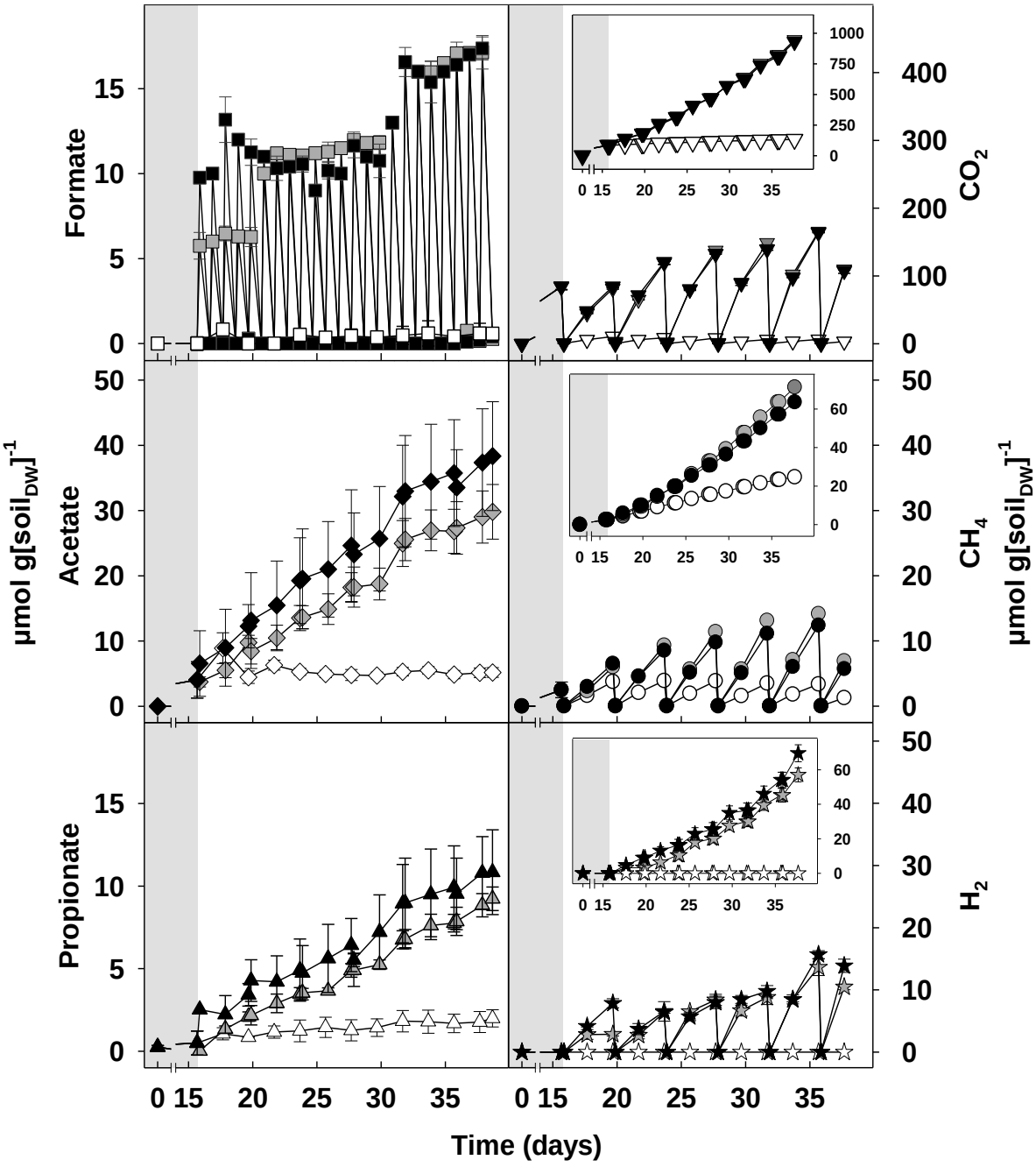


Figure 28: Effect of supplemental [^{13}C]formate on the production of organic acids and gases in 'soil' slurries.

'Soil' was obtained from mire 2 and anoxic slurries were incubated at 15 °C. Shaded area indicates period of preincubation. CO_2 in formate-supplemented slurries is the combined CO_2 from the bicarbonate pulses and CO_2 derived from the apparent conversion of formate to H_2 and CO_2. Symbols: open symbols, unsupplemented controls; grey symbols, [^{12}C]formate-supplemented slurries; closed symbols, [^{13}C]formate-supplemented slurries. The gas phase was periodically exchanged with 100 % N_2. Inserts show cumulative gas concentrations. Values are the means of triplicate slurries and the error bars indicate standard deviation. Figure was modified from Hunger *et al.* (2011a).

Formate-dependent production of H_2 occurred before the formate-dependent production of acetate and CH_4, underlying the rapid conversion of formate to H_2 and CO_2 by FHL-containing taxa. Consumption of H_2 was not observed, indicating that formate was the main driver for the production of acetate and CH_4. Approximately 17 at % and 1 at % of acetate-derived carbon were enriched with ^{13}C in [^{13}C]formate and [^{12}C]formate-supplemented slurries, respectively, reinforcing the likelihood that acetogens participated in the synthesis of acetate in formate-supplemented slurries.

Table 32: Recovery of reductant and carbon from supplemental formate after 23 days of supplementation.[a]

	Reductant recovered from formate (%)		Carbon recovered from formate (%)	
Products	[^{13}C]formate	[^{12}C]formate	[^{13}C]formate	[^{12}C]formate
Acetate	46	38	23	19
Propionate	22	18	9	8
CH_4	54	70	14	18
H_2	24	21	n.a.	n.a.
Total:	146	147	46	45

[a] Recovery was calculated based on process data at the beginning and after 23 days of formate supplementation (Figure 28). CO_2 was not considered in the calculation because it was regularly pulsed in form of bicarbonate and also removed by the exchange of the gas phase. Values are rounded to nearest whole number. Abbreviation: n.a., not applicable.

Product profiles of ^{13}C and ^{12}C treatments (Figure 28, Table 32) were very similar, indicating that similar microbial activities occurred in these treatments. Most reducing equivalents and carbon from supplemental formate were recovered in CH_4 and acetate (Table 32). Recovery of supplemental formate-derived reductant exceeded 100 % in both [^{13}C]formate and [^{12}C]formate-supplemented slurries. A recovery greater than 100 % suggested that supplemental formate enhanced the use of endogenous substrates (i.e., priming effect) which has been observed in other studies (Fontaine *et al.* 2004, Guenet *et al.* 2010, Schellenberger *et al.* 2010).

5.3.2 Bioenergetics of formate-dependent acetogenesis

The estimated Gibbs free energy of the apparent formate-dependent acetogenesis in [^{13}C]formate-supplemented slurries averaged -42 kJ mol^{-1} acetate (Figure 29), indicating that this process was thermodynamically feasible under the experimental conditions.

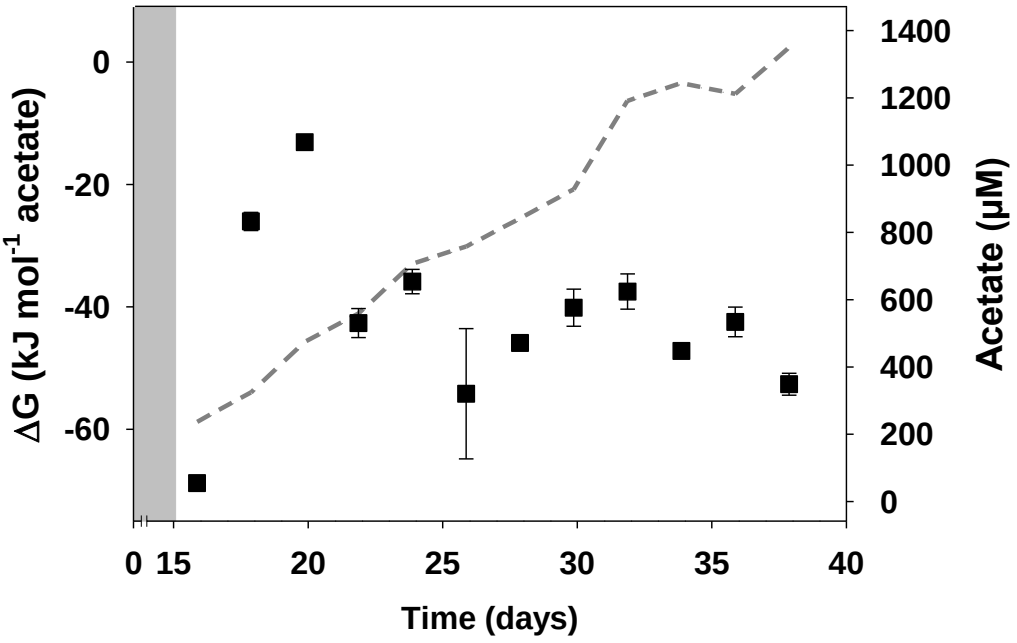


Figure 29: Estimated Gibbs free energy (∆G) of formate-dependent acetogenesis in [^{13}C]formate-supplemented 'soil' slurries.

Shaded area indicates period of preincubation. Filled squares and dashed line show values of ∆G for formate-dependent acetogenesis and concentration of acetate, respectively. Values are the means of triplicate slurries and the error bars indicate standard deviation. For process data see Figure 28.

5.3.3 Diversity of bacterial *fhs* phylotypes

After the isopycnic centrifugation of DNA in a cesium chloride gradient and subsequent fractionation (4.10.7), heavy DNA from fraction 4 was used for the molecular analysis of *mcrA*, and bacterial and archaeal 16S rRNA genes (for details see Hunger *et al.* 2011a), but fraction four did not yield a PCR signal for *fhs*. Thus, *fhs* sequences were obtained from DNA of fractions five and seven (Figure 30).

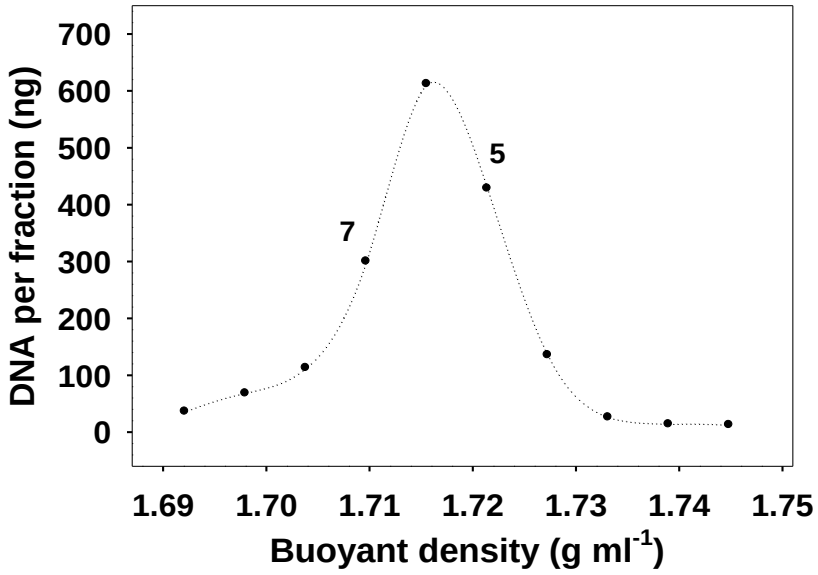


Figure 30: Distribution of DNA from [^{13}C]formate-supplemented 'soil' slurries in a cesium chloride gradient.

DNA derived from anoxic slurries after 23 days of formate supplementation (Figure 28). Numbers indicate fractions used for molecular analysis. Figure was modified from Hunger *et al.* (2011a).

A total of 70 bacterial *fhs* sequences were obtained that clustered into 13 species-level phylotypes (Table 33). Rarefaction curves and coverage indicated that sampling was sufficient for species-level clustering of *fhs* phylotypes in the heavy fraction derived from [^{13}C]formate-supplemented slurries, but not in the other two gene libraries (Figure 31, Table 33).

Table 33: Coverage of clone libraries, number of *fhs* sequences, and number of species-level *fhs* phylotypes obtained from [^{13}C]formate- and [^{12}C]formate-supplemented 'soil' slurries.

Gene	Clone libraries[a]	No. of sequences	No. of phylotypes	Coverage
fhs	Heavy fraction [^{13}C]formate[b]	44	7	>99
	Light fraction [^{13}C]formate[b]	10	8	40
	Heavy fraction [^{12}C]formate[c]	16	7	88
	Total:	70	13	93

[a] Heavy fraction is fraction five and light fraction is fraction seven of a cesium chloride gradient (DNA SIP). For process data see Figure 28.
[b] Sequences derived from anoxic [^{13}C]formate-supplemented slurries after 23 days of anoxic incubation.
[c] Sequences derived from anoxic [^{12}C]formate-supplemented slurries after 23 days of anoxic incubation.

The detected species-level *fhs* phylotypes were affiliated with *Alphaproteobacteria*, *Clostridia*, *Planctomycea*, and *Holophagae* (Figure 32). Species-level phylotype PTL2 was

only observed in the heavy fraction derived from [^{13}C]formate-supplemented slurries, and may potentially be labeled. Species-level phylotype PTL4 had a relative abundance of 45 % in the heavy fraction derived from [^{13}C]formate-supplemented slurries. PLT4 was not detected in the light fraction derived from [^{13}C]formate-supplemented slurries and had a low relative abundance in the heavy fraction derived from [^{12}C]formate-supplemented slurries, suggesting a potential labeling of associated taxa. Unfortunately, it remains mostly unknown which *fhs* phylotypes might be labeled since (a) too few sequences were obtained from the heavy fraction derived from [^{12}C]formate-supplemented slurries and the light fraction derived from [^{13}C]formate-supplemented slurries, and (b) analyzed light and heavy fractions were insufficiently separated in the cesium chloride gradient.

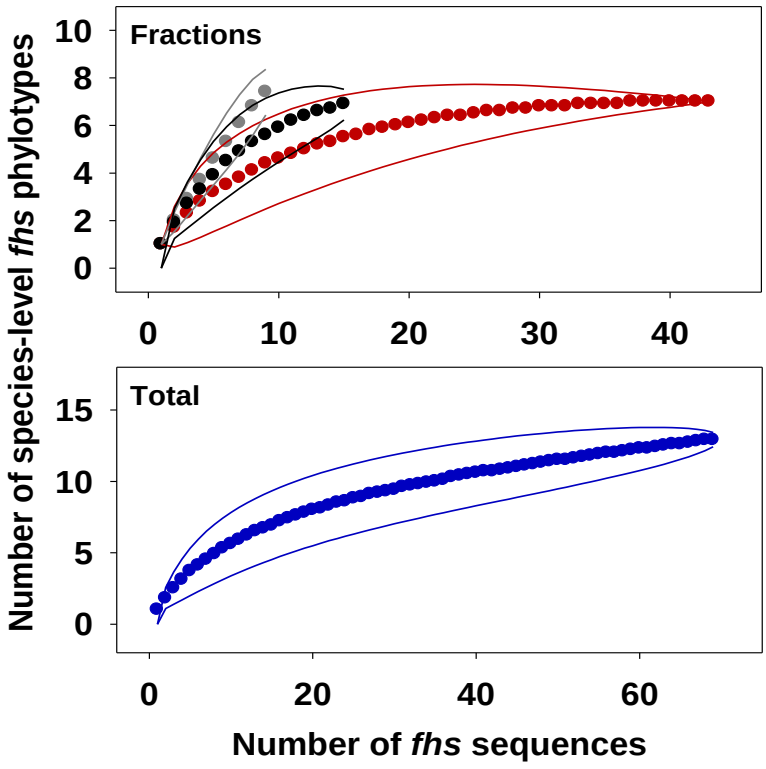


Figure 31: Rarefaction analysis of species-level *fhs* phylotypes obtained from [^{13}C]formate- and [^{12}C]formate-supplemented 'soil' slurries.

Sequences were obtained from heavy fraction five and light fraction seven after isopycnic centrifugation of DNA derived from anoxic formate-supplemented 'soil' slurries (Figure 28). *fhs* sequences were *in silico* translated into amino acids and based on a 76.4 % similarity cut-off clustered into species-level phylotypes (5.1.2). 95 % confidence intervals are shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Color code: red, heavy fraction of [^{13}C]formate-supplemented slurries; grey, light fraction of [^{13}C]formate-supplemented slurries; black, heavy fraction of [^{12}C]formate-supplemented slurries; blue, total number of sequences.

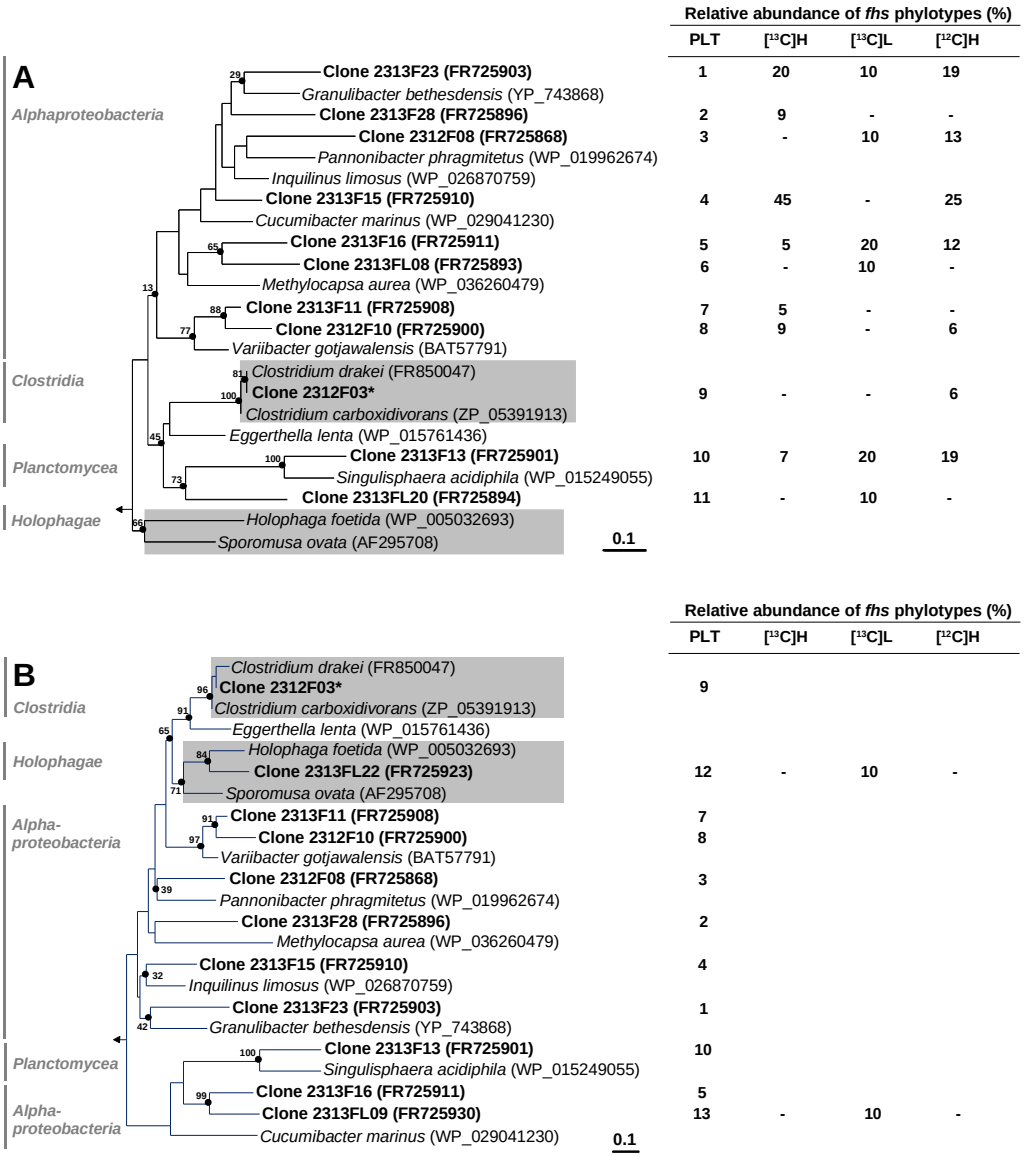


Figure 32: Phylogenic maximum parsimony trees of (a) representative *fhs*-encoded amino acid sequences retrieved from formate-supplemented 'soil' slurries and (b) reference sequences.

Sequences derived from heavy fraction five and light fraction seven of [^{13}C]-enriched DNA (Figure 30) obtained from [^{13}C]formate-supplemented 'soil' slurries after 23 days of supplementation (Figure 28). *fhs* sequences were *in silico* translated into amino acids and based on a 76.4 % similarity cut-off clustered into species-level phylotypes (5.1.2). Accession numbers are indicated in brackets. Sequences in Panel A correspond to residues 199-334 of the *fhs*-encoded amino acid sequence of *Clostridium difficile* 630 (NC_009089). Sequences of Panel B correspond to residues 292-407 of the *fhs*-encoded amino acid sequence of *Clostridium difficile* 630 (NC_009089). Filled dots at nodes indicate the confirmation of tree topology by three calculations with the same data set (neighbor joining, maximum likelihood, and maximum parsimony algorithms). *Methanocorpusculum labreanum* (CP000559) was used as outgroup. Bar indicates a 0.1 estimated change per amino acid. Bootstrap values derived from the maximum parsimony tree (1,000 resamplings) are only displayed at nodes congruent in all three trees. Grey highlights indicate sequences of acetogens. Legend: [^{13}C]H, heavy fraction from [^{13}C]formate-supplemented slurries; [^{13}C]L, light fraction from [^{13}C]formate-supplemented slurries; [^{12}C]H, heavy fraction from [^{12}C]formate-supplemented slurries; PLT, phylotypes; *, sequence was not submitted to EMBL and thus has no accession number. Values are rounded to nearest whole number. Figure was modified from Hunger *et al.* (2011a).

Four out of 13 species-level *fhs* phylotypes included cultured isolates. Phylotypes PLT7 (85 % amino acid sequence similarity) and PLT8 (79 % amino acid sequence similarity) were most closely related to *Variibacter gotjawalensis* (BAT57791) (Figure 32). Phylotypes PLT9 (99 % amino acid sequence similarity) and PLT12 (77 % amino acid sequence similarity) were most closely related to the acetogens *C. drakei* (FR850047) and *Holophaga foetida* (WP_005032698), respectively.

5.4 Anaerobic processes associated with the root zone of mire-derived plants

5.4.1 Effect of supplemental formate on product profiles of root-free soil and soil-free root slurries

Formate can be utilized by mire methanogens and can be released from roots of wetland plants (Hunger *et al.* 2011a, Koelbener *et al.* 2010). Although formate was anticipated to directly trigger methanogenesis, the production of CH_4 by anoxic slurries of soil-free roots obtained from *Carex rostrata*, *Carex nigra*, and *Molinia caerulea* was not stimulated by an initial pulse of formate (Figure 33, Figure 34). Unsupplemented roots of *C. rostrata*, *C. nigra*, and *M. caerulea* initially formed H_2 from endogenous sources. The initial pulse of formate was rapidly consumed with the concomitant production of H_2 and CO_2 in a stoichiometric ratio of consumed formate to produced H_2 of 1:0.9, 1:1.6, and 1:2 with roots of *C. rostrata*, *C. nigra*, and *M. caerulea*, respectively. The rapid consumption of formate indicated that root-associated microorganisms were poised to consume formate and that produced H_2 derives from formate and other endogenous sources. In this regard, up to 10 µmol formate per $g[root_{DW}]^{-1}$ was detected with fresh *Carex* roots at the onset of unsupplemented incubations, a finding consistent with the release of formate from the roots of wetland plants (Koelbener *et al.* 2010). The production of H_2 in response to the initial pulse of formate was transient with roots from *C. rostrata* and *M. caerulea*, and the subsequent consumption of formate-derived H_2 was concomitant with an increased production of CH_4 and acetate with *C. rostrata* roots (Figure 33), activities indicative of hydrogenotrophic methanogenesis and acetogenesis, respectively.

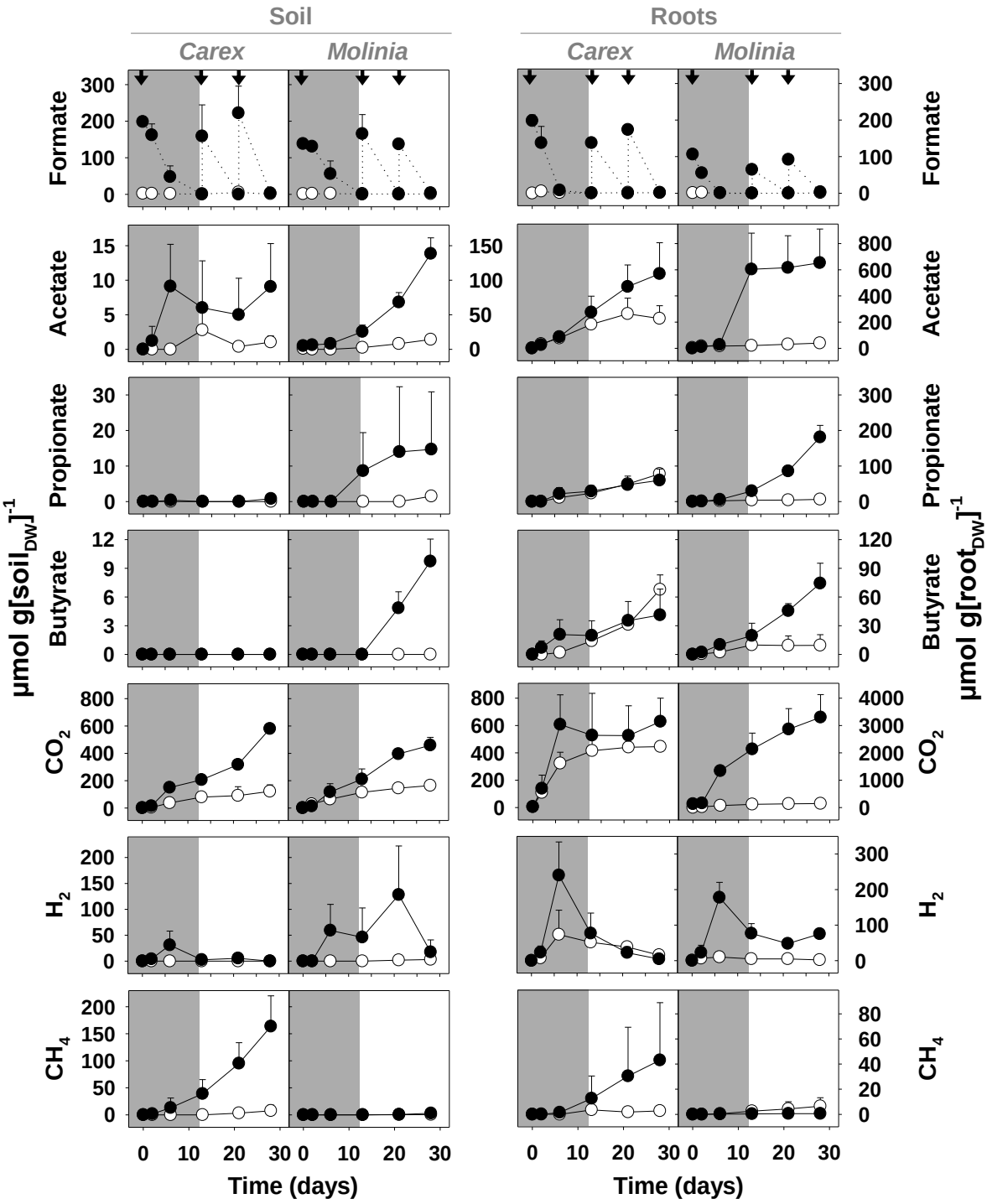


Figure 33: Effect of formate pulses on product profiles of soil-free roots and root-free soils of *C. rostrata* and *M. caerulea*.

Roots or soils were obtained from mire 2. Roots or soils were diluted 1:10 with anoxic solution, incubated in anoxic flasks at 15 °C, and pulsed repeatedly (arrows) with approximately two millimolar formate. Symbols: open symbols, unsupplemented controls; closed symbols, formate-supplemented slurries. Shaded area represents the period corresponding to the first pulse of formate. Values are means with the standard deviation of triplicate analysis for formate treatments and duplicate analysis for controls. Figure was modified from Hunger *et al.* (2016).

The subsequent consumption of formate-derived H_2 with *M. caerulea* roots was concomitant with an increased production of acetate, propionate, and butyrate but not CH_4, activities indicative of hydrogenotrophic acetogenesis and other processes. Formate-derived H_2 accumulated with roots from *C. nigra*, and accumulation ceased after the consumption of the third formate pulse (Figure 34), indicating a positive correlation between formate consumption and H_2 production by FHL-containing taxa. The production of acetate in formate-supplemented slurries with *C. nigra* roots was negligibly elevated in comparison with the unsupplemented control after secondary and tertiary pulses of formate, suggesting that acetogenesis and other acetate-producing anaerobic processes (such as fermentation) were mostly substrate saturated.

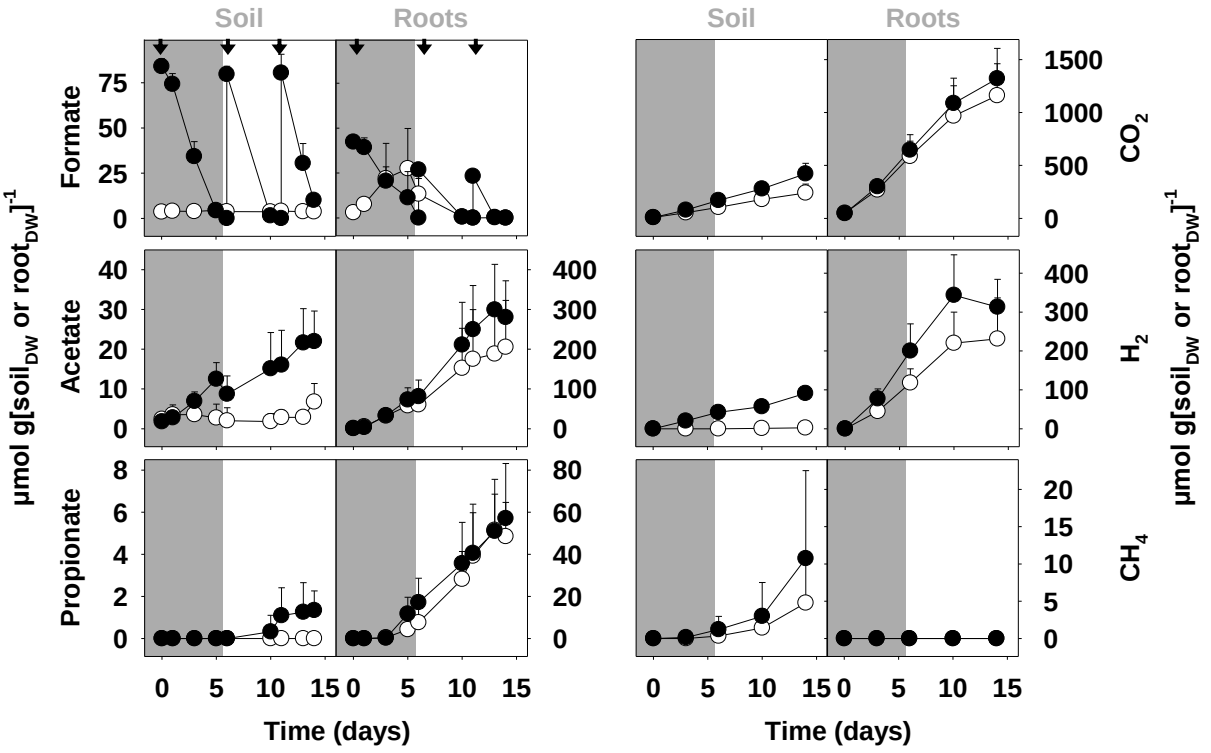


Figure 34: Effect of formate pulses on product profiles of soil-free roots and root-free soils of *C. nigra*.

Roots or soils were obtained from mire 2. Roots or soils were diluted 1:10 with anoxic solution, incubated in anoxic flasks at 15 °C, and pulsed repeatedly (arrows) with approximately one millimolar formate. Symbols: open symbols, unsupplemented controls; closed symbols, formate-supplemented slurries. Shaded area represents the period corresponding to the first pulse of formate. Values are means with the standard deviation of sextuplicate analysis. Data plotted in this figure was obtained by Madena Eppendorfer as part of her master thesis.

The consumption of secondary and tertiary pulses of formate did not yield H_2 but was concomitant with the increased production of CH_4 and acetate in slurries with *C. rostrata* roots (Figure 33), indicating (a) that the consumption of formate-derived H_2 was tightly coupled to methanogenesis and acetogenesis, or (b) that formate was directly dissimilated by methanogens and acetogens. In contrast to *C. rostrata roots*, secondary and tertiary formate pulses with *M. caerulea* roots stimulated not only the production of acetate but also the production of propionate, butyrate, and H_2, but not CH_4.

Slurries with root-free soil from the same patches where corresponding plant roots derived from were in general much less active than root slurries (Figure 33, Figure 34). Except of the production of CH_4 which is higher in slurries with soils from *C. rostrata* and *C. nigra* than in corresponding root slurries. CH_4 was not detected with 'soil' slurries from *M. caerulea*. Initial formate pulses slightly stimulated the production of H_2, CO_2 and acetate in slurries with soil from *C. rostrata*, *C. nigra*, and *M. caerulea*. Secondary and tertiary formate pulses slightly stimulated the production of propionate and butyrate in slurries with soil from *M. caerulea* which has also been observed with corresponding roots to a higher degree (Figure 33).

The collective amount of reductant in the additional CH_4 and acetate formed exceeded what was available in the consumed supplemental formate, suggesting that formate had a 'priming' effect (Fontaine *et al.* 2004) on the consumption of endogenous organic carbon, an activity reported earlier with root-containing peat soil obtained from the same mire (5.3.1).

5.4.2 Effect of increasing formate supplementation on product profiles of *Carex* root and soil slurries.

The aforementioned findings unexpectedly indicated that *Carex* and *Molinia* roots had a high initial capacity for the anaerobic transformation of formate to H_2 by enzymes such as FHL. This anaerobic activity was examined in more detail by determining the initial response of roots and soils to increasing concentrations of formate. Increasing amounts of supplemental formate yielded a rapid increase in the amounts of H_2 produced by soil-free roots of *Carex* sp. (Figure 35). Pulses with increasing amounts of supplemental formate had a marginal effect

on (a) H_2 and CH_4 production on *Carex* soil and (b) the production of CH_4 on *Carex* roots. Based on the amount of supplemental formate (which was totally consumed in each treatment) and correcting for the amount of H_2 formed in unsupplemented controls, formate:H_2 ratios were approximately 1:1, thus confirming the activity of FHL-containing taxa located on *Carex* roots.

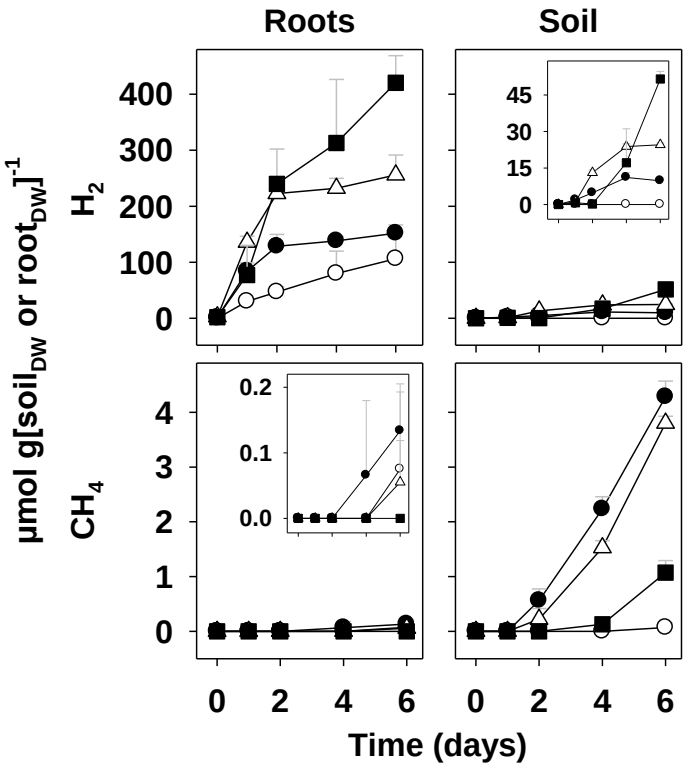


Figure 35: Effect of supplemental formate on the formation of H_2 and CH_4 in slurries with soil-free roots and root-free soils obtained from *Carex*.

Roots or soils were obtained from *Carex* sp. (potentially *Carex nigra*, *Carex rostrata*, and *Carex canescens*) from mire 2. Roots and soils were diluted 1:10 with anoxic solution and incubated in anoxic flasks at 15 °C. Symbols: open circle, unsupplemented controls; closed circle, supplemented with approximately 70 µmol formate g_{DW}^{-1}; open triangle, supplemented with approximately 180 µmol formate g_{DW}^{-1}; closed square, supplemented with approximately 390 µmol formate g_{DW}^{-1}. Values are means with the standard deviation of triplicate analysis. Figure was modified from Hunger *et al.* (2016).

5.4.3 Formation of gases with unsupplemented soil-free roots and root-free soil from *Carex*

Slurries of unsupplemented *Carex* roots had the potential to produce H_2 under anoxic conditions (Figure 33, Figure 34). The anaerobic capacity to produce H_2 was also observed with soil-free roots that were incubated without the addition of the anoxic solution used to make slurries (Figure 36). The same trend was observed with CO_2. Root-free soil had the capacity

to produce more CH_4 in comparison to soil-free roots without the addition of anoxic solution, an observation that has been made with slurries of roots and soils (Figure 33, Figure 34, Figure 35). Thus, those root- and soil-associated activities were independent of the slurry condition. H_2 was not detected in incubations of unsupplemented non-slurries of root-free soil (Figure 36), a result consistent with that observed with root-free soil slurries (Figure 33, Figure 34, Figure 35).

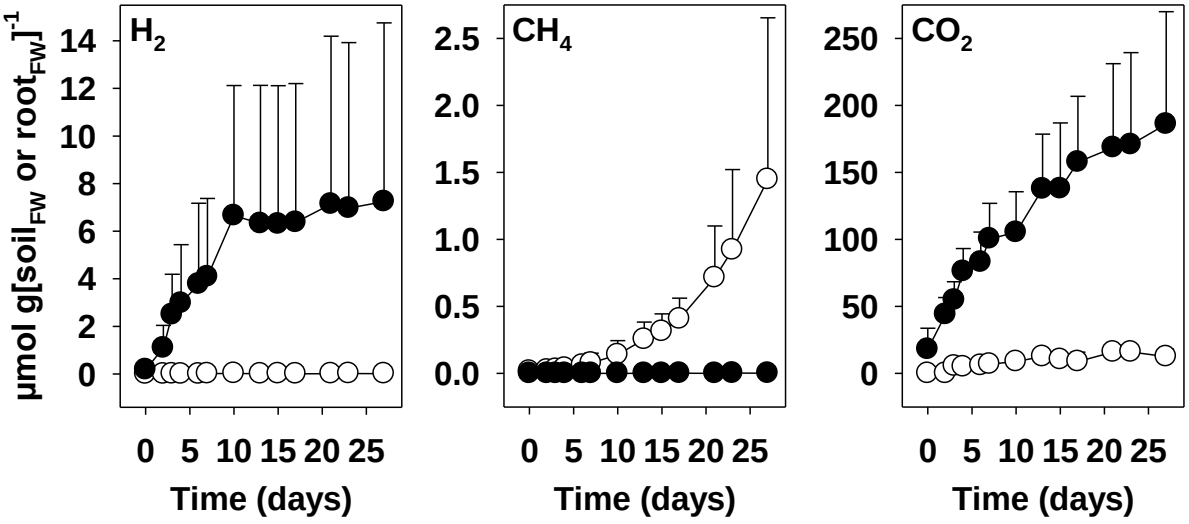


Figure 36: Formation of gases from soil-free roots and root-free soils obtained from *Carex* sp.

Roots or soils were obtained from *Carex* sp. (potentially *C. nigra*, *C. rostrata*, and *C. canescens*) from mire 2. Roots or soils were incubated in anoxic flasks without the addition of anoxic solution and incubated at 15 °C. Symbols: closed circle, unsupplemented soil-free roots; open circle, unsupplemented root-free soils. Values are means with standard deviation of sextuplicate analysis. Figure was modified from Hunger *et al.* (2016).

5.4.4 Bioenergetics of anaerobic formate- and H_2-driven processes

Calculated Gibbs free energy for acetogenesis, methanogenesis, and the formation of formate-derived H_2 by enzymes such as FHL were mostly negative in slurries with roots from *C. rostrata* and *M. caerulea*, and soils from the same plant patches (Figure 37), indicating that these processes were thermodynamically feasible during the incubations. H_2- and formate-dependent methanogenesis was more negative than H_2- and formate-dependent acetogenesis and also more negative than the formation of formate-derived H_2, suggesting that these H_2- and formate-driven processes had different thermodynamic potentials.

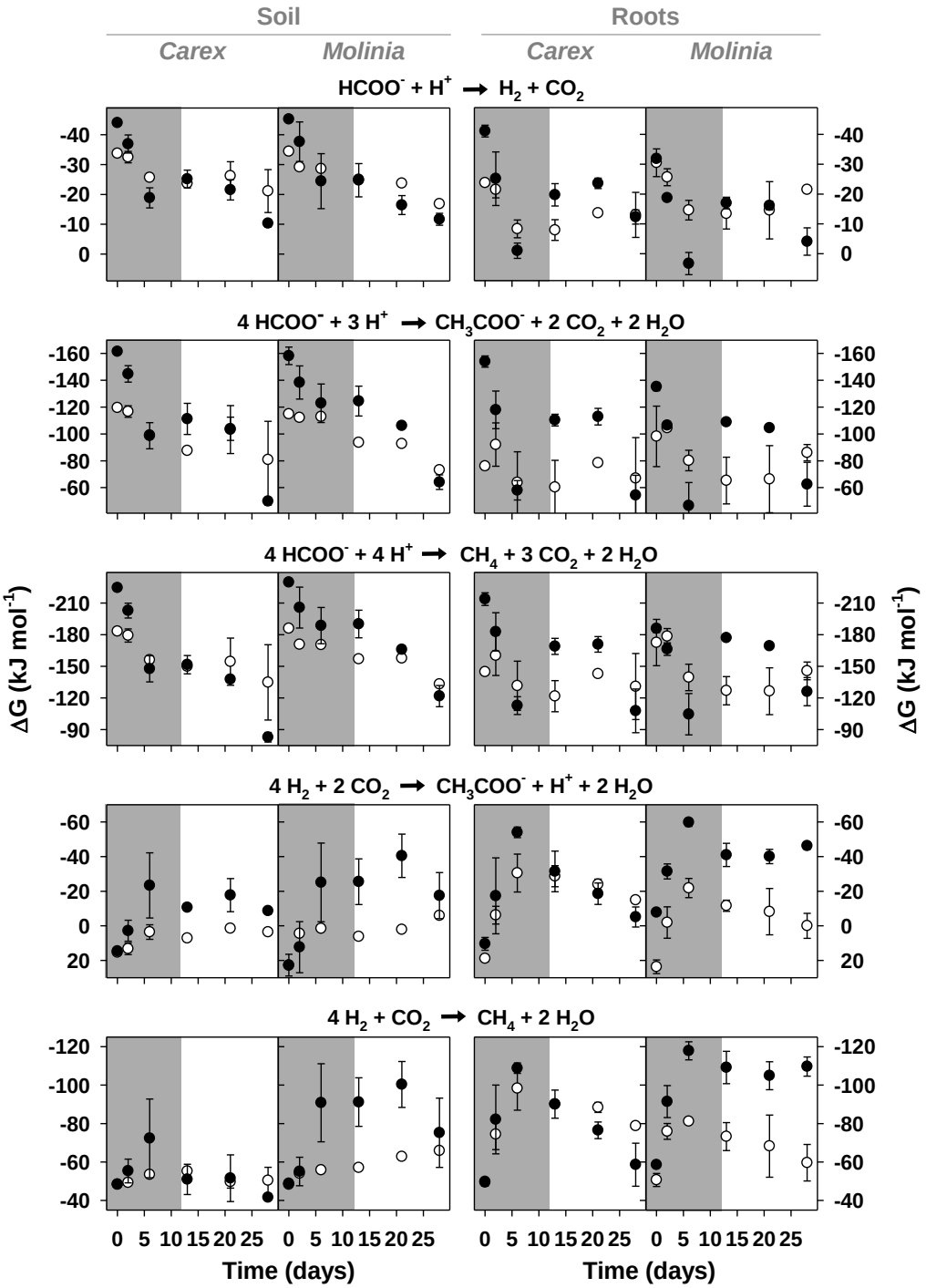


Figure 37: Calculated Gibbs free energy (∆G) in slurries with soil-free roots and root-free soils from *C. rostrata* and *M. caerulea*.

The following concentrations and partial pressures were assumed when the corresponding substances could not be detected: 5 Pa for H_2, 15 Pa for CO_2, 0.5 Pa for CH_4, one micromolar for formate and one micromolar for acetate. This was necessary to avoid division by zero during calculations. Process data of incubations are in Figure 33 and shaded area represents the period corresponding to the first pulse of formate. Symbols: open symbols, unsupplemented controls; closed symbols, formate-supplemented slurries. Values are means with standard deviation of triplicate analysis for formate treatments and duplicate analysis for controls. Figure was modified from Hunger *et al.* (2016).

5.4.5 Diversity of *mcrA*/*mrtA* phylotypes of *C. rostrata* roots

McrA and *mrtA* encode for the alpha-subunit of the isoenzyme I and II of the methyl-CoM reductase, respectively, enzymes that catalyze the terminal step in methanogenesis (Thauer 1998, Pihl *et al.* 1994). In total, 174 *mcrA*/*mrtA* sequences (including two *mrtA* sequences) obtained from *C. rostrata* roots clustered into nine species-level phylotypes (Table 34). Rarefaction analysis indicated the diversity of *mcrA*/*mrtA* phylotypes of roots were similar before and after incubation, and coverage indicated that sampling was adequate for species-level determination (Table 34, Figure 38).

Table 34: Coverage of clone libraries, number of *mcrA*/*mrtA* sequences, and number of species-level *mcrA*/*mrtA* phylotypes obtained from *C. rostrata* roots.

Gene	Clone libraries[a]	No. of sequences	No. of phylotypes	Coverage
mcrA/*mrtA*	Roots before incubation	58	8	97
	Unsupplemented roots	59	6	>99
	Formate-supplemented roots	57	6	97
	total:	174	9	>99

[a] Unsupplemented roots and formate-supplemented roots were analyzed after 28 days of incubation (Figure 33).

Methanosarcinaceae, *Methanoregulaceae*, and *Methanobacteriaceae* were the most abundant phylotypes detected before and after incubation of *C. rostrata* roots (Figure 39), indicating that hydrogenotrophic and aceticlastic methanogens were the most dominant taxa on *C. rostrata* roots and in root slurries after incubation. *Methanoregulaceae* was the most abundant taxon before incubation of *C. rostrata* roots and decreased in relative abundance due to incubation, indicating that incubation conditions did not favor *Methanoregulaceae*-associated methanogens.

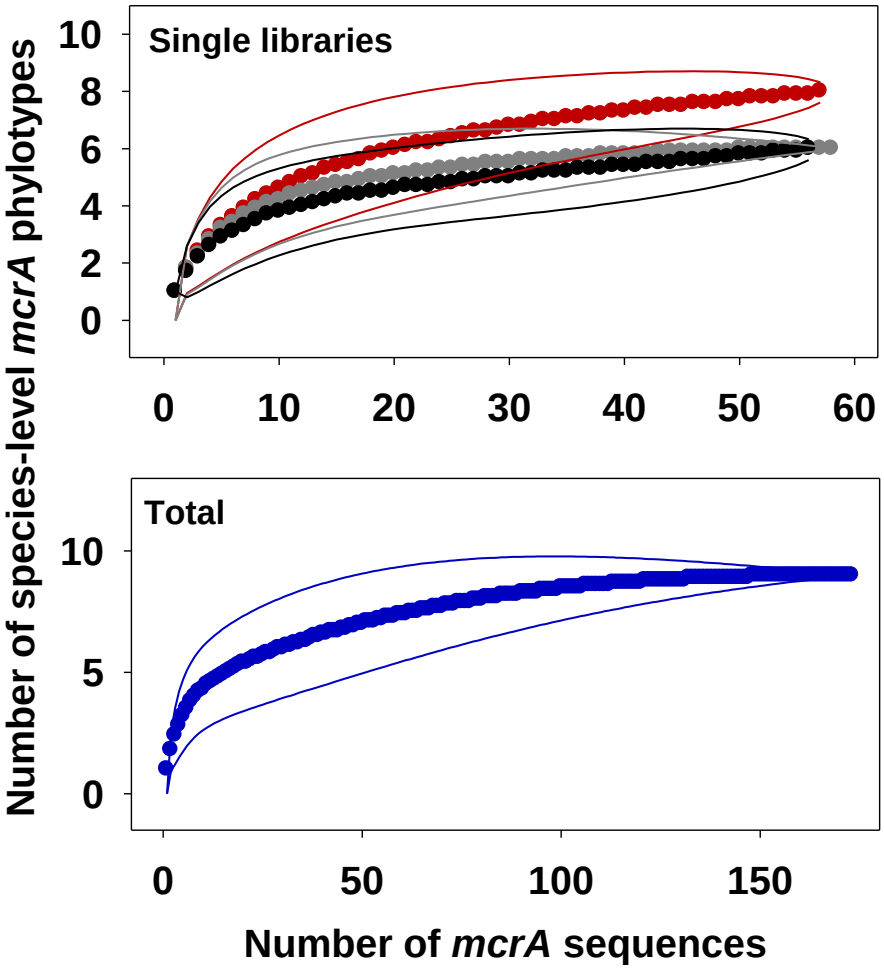


Figure 38: Rarefaction analysis of species-level *mcrA*/*mrtA* phylotypes obtained from *C. rostrata* roots.

Sequences were obtained from roots before and after 28 days of incubation (Figure 33). *mcrA*/*mrtA* sequences were *in silico* translated into amino acids and based on an 85.7% similarity cut-off clustered into species-level phylotypes (5.1.2). 95 % confidence intervals are shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Color code: red, roots without treatment; grey, unsupplemented roots; black, formate-supplemented roots; blue, total number of sequences. Figure was modified from Hunger *et al.* (2016).

Methanobacteriaceae was the most abundant taxon in unsupplemented controls after incubation and showed a lower relative abundance before incubation and after formate supplementation, indicating that incubation conditions supported growth of *Methanobacteriaceae*-affiliated methanogens but formate and/or products of formate consumption (e.g., accumulating acetate) inhibited those taxa.

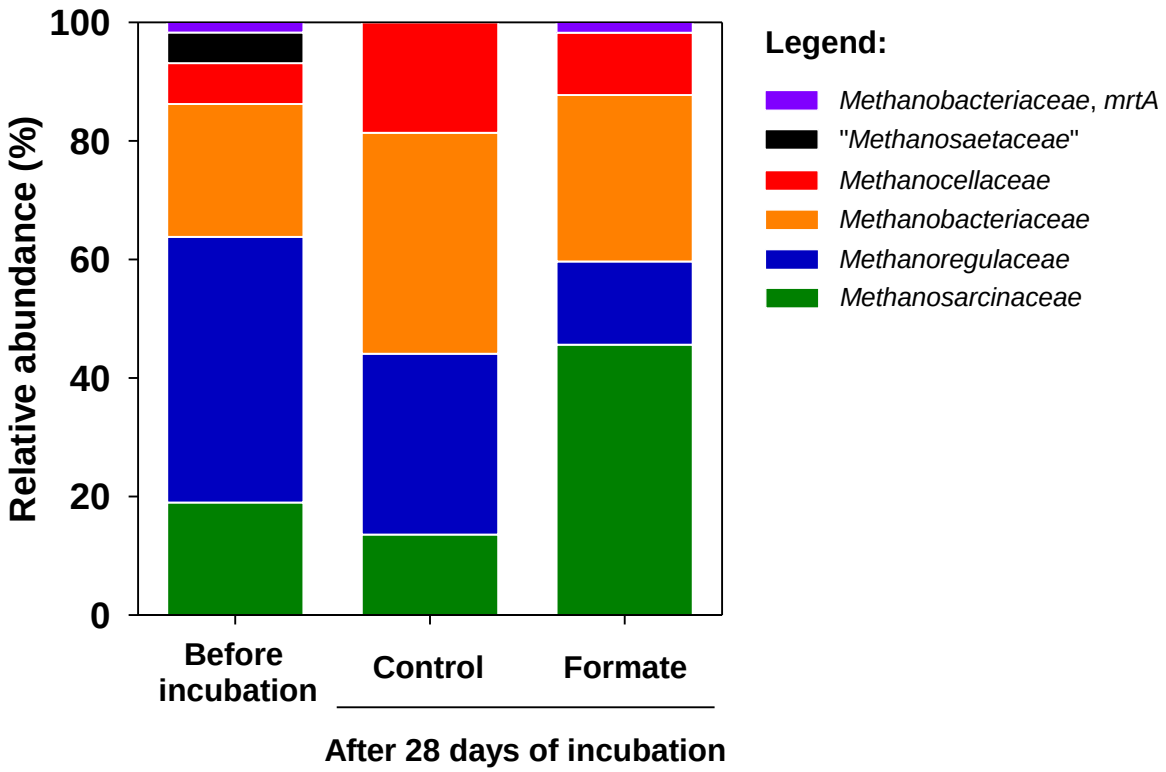
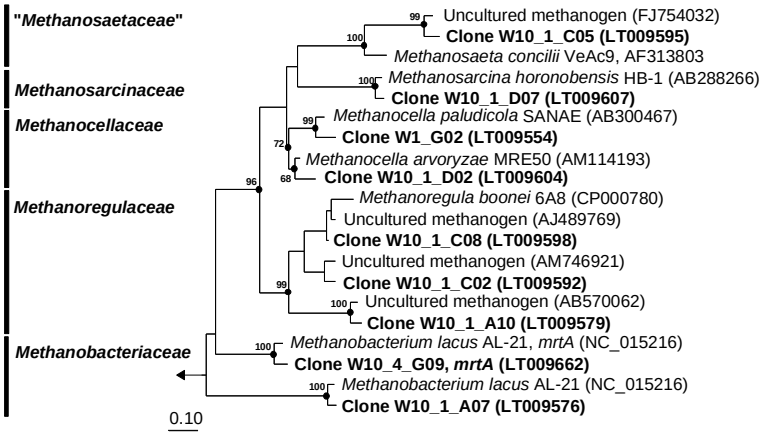


Figure 39: Relative abundances of family-level *mcrA*/*mrtA* phylotypes from *C. rostrata* roots.

The plot shows family-level *mcrA* phylotypes and one family-level *mrtA* phylotype. Process data of incubations are in Figure 33. *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net).

Methanosarcinaceae (consists of a *Methanosarcina horonobensis*-affiliated species-level phylotypes, Figure 40) increased in relative abundance during incubation with supplemental formate, indicating that *Methanosarcina*-affiliated methanogens favored conditions in formate-supplemented slurries (e.g., high acetate and H_2 concentrations). *Methanocellaceae* and "*Methanosaetaceae*" accounted collectively for not more than 19 %, indicating a relatively minor importance to the production of CH_4.



	Relative abundance of sequences (%)			
	Before	After 28 days		
PLT	incubation	Control	Formate	Total
1	5	-	-	2
2	19	14	46	26
3	-	3	-	1
4	7	15	11	11
5	2	3	2	2
6	38	27	12	26
7	5	-	-	2
8	2	-	2	1
9	22	38	28	29

Figure 40: Phylogenic maximum parsimony tree of (a) representative *mcrA*/*mrtA*-encoded amino acid sequences retrieved from *C. rostrata* roots and (b) reference sequences.

Sequences were obtained from roots before and after 28 days of incubation (Figure 33). *mcrA*/*mrtA* sequences were *in silico* translated into amino acids and based on an 85.7 % similarity cut-off clustered into species-level phylotypes (5.1.2). *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net). Accession numbers are indicated in brackets. Sequences correspond to residues 98-227 of the *mcrA* amino acid sequence of *M. paludicola* (AB300467). Filled dots at nodes indicate the confirmation of tree topology in three calculations with the same data set (maximum parsimony, neighbor joining, and maximum likelihood algorithms). *M. kandleri* (AE009439) was used as outgroup. Bar indicates a 0.1 change per amino acid. Bootstrap values are averages from the maximum parsimony tree (1,000 resamplings), the neighbor joining tree (1,000), and the maximum likelihood tree (100) and are only displayed at nodes congruent in all three trees. Legend: PLT, phylotypes; -, not detected. Values are rounded to the nearest whole number. Figure was modified from Hunger *et al.* (2016).

5.4.6 Diversity of bacterial 16S rRNA gene sequences in an early root-derived enrichment

Roots of *C. rostrata*, *C. nigra*, and *M. caerulea* showed the capacity of formate-driven production of H_2 and acetate (Figure 33, Figure 34). Thus, a mixture of *Carex* and *Molinia* roots from mire 2 was used to enrich for bacterial taxa responsible for formate consumption. On a per liter basis, an early root enrichment consumed approximately 54 ± 6 mmol formate and 87 ± 3 mmol H_2 as co-substrates and produced approximately 36 ± 2 mmol acetate at a pH of approximately five in the presence of BES. In theory, 54 mmol formate and 87 mmol H_2 would collectively yield approximately 35 mmol acetate via acetogenesis. Therefore, the substrate-product ratio of the early root enrichment was in strong evidence of acetogenesis. BES was added to the enrichment to inhibit methanogenic archaea. Formate- and H_2-

consuming bacteria were identified by 16S rRNA gene analysis of this early root-derived enrichment.

Table 35: Coverage of clone libraries, number of 16S rRNA gene sequences, and number of family-level 16S rRNA gene phylotypes obtained from mire-derived roots.[a]

Gene	Clone libraries	No. of sequences	No. of phylotypes	Coverage
16S rRNA gene	Before incubation[b]	41	14	83
	Unsupplemented control[c]	47	14	83
	Formate-H_2-supplementation[c]	43	16	77
	Total:	131	31	90

[a] Sequences derived from a mixture of *M. caerulea* and *Carex* sp. roots from mire 2.
[b] Sequences were obtained from roots before incubation.
[b] Sequences were obtained from roots after 23 days of anoxic incubation (i.e., the early enrichment).

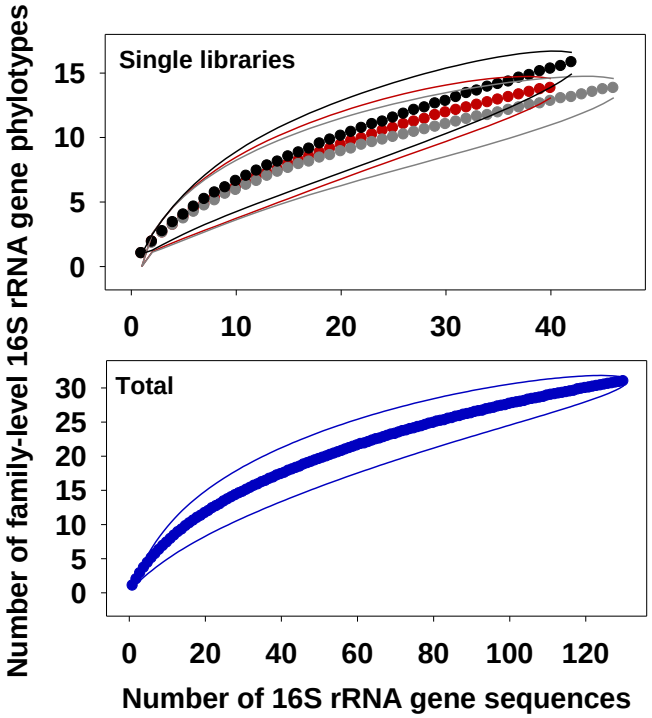


Figure 41: Rarefaction analysis of family-level 16S rRNA gene phylotypes obtained from mire-derived roots.

Sequences were obtained from a mixture of *M. caerulea* and *Carex* sp. roots before and after 23 days of anoxic incubation. Assignment of 16S rRNA gene sequences to family-level phylotypes was based on an 87.5 % similarity cut-off (Yarza *et al.* 2008). 95 % confidence intervals are shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Color code: red, roots before incubation; grey, unsupplemented roots; black, formate-H_2-supplemented roots; blue, total number of sequences.

In total, 131 16S rRNA gene sequences were detected with samples from the early enrichment, which clustered into seven phylum-level phylotypes and 31 family-level phylotypes (Table 35). Rarefaction curves and coverage indicated that sampling was sufficient for family-level clustering of 16S rRNA gene phylotypes (Table 35, Figure 41). The diversity of family-level phylotypes was similar on roots before and after the incubation and independent of supplementation (Figure 41).

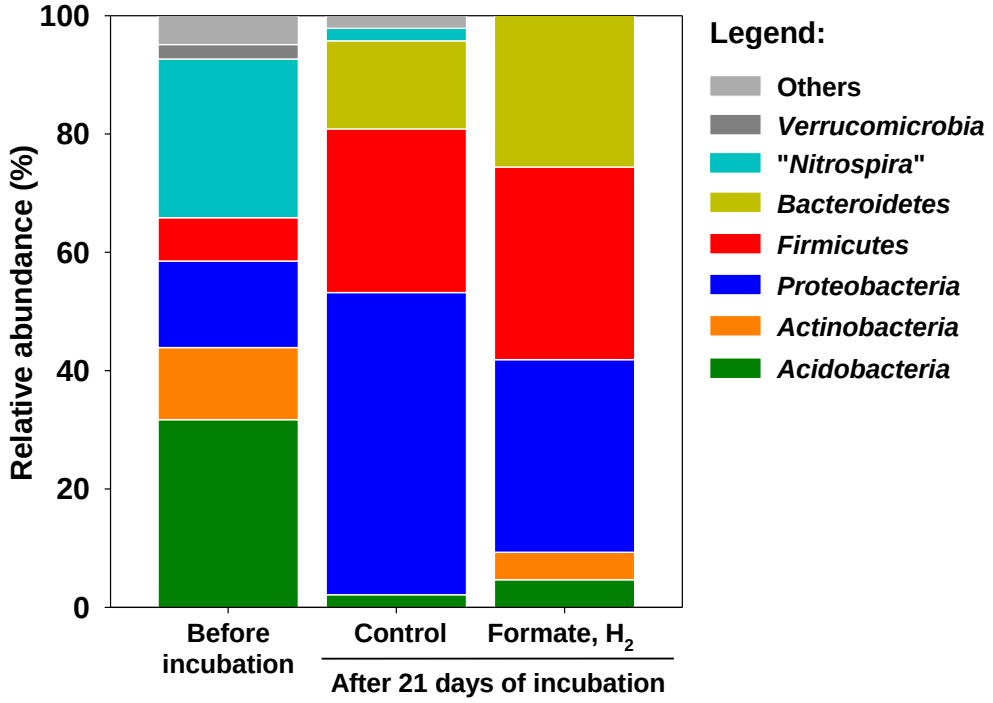


Figure 42: Relative abundance of phylum-level 16S rRNA gene phylotypes of mire-derived roots.

Sequences derived from a mixture of *M. caerulea* and *Carex* sp. roots before and after 23 days of anoxic incubation. "Others" include all sequences that could not be assigned to known phyla.

Acidobacteria and "*Nitrospira*" were the most abundant phyla before incubation of roots and accounted for 31 % and 27 % but decreased in relative abundance during incubation (i.e., to approximately 4 % and 2 %), respectively (Figure 42), suggesting that affiliated taxa were abundant on roots before the incubation but were not stimulated by conditions of the incubation. The relative abundance of *Proteobacteria* and *Firmicutes* increased during incubation from 14 % and 7 % to 51 % and 28 % with unsupplemented roots and 32 % and 32 % with formate-supplemented roots, respectively, indicating that incubation conditions favored growth of *Proteobacteria*- and *Firmicutes*-affiliated bacteria.

16S rRNA gene sequences closely related to the acetogens *Clostridium drakei* (98 % sequence similarity, Y18813) and *Clostridium scatologenes* (98 % sequence similarity, AB601088) were detected on roots before incubation and relative abundance of those *Clostridium*-affiliated sequences increased during supplementation with formate and H_2 (Figure 43), indicating that *Clostridium*-affiliated root-derived acetogens might utilize formate and H_2.

Table 36: Taxonomic identities and relative abundances of 16S rRNA gene phylotypes of mire-derived roots.

	Relative abundance (%)[a]		
		After 21 days	
Taxonomic level (phylum, class, family)	Before incubation	Control	Formate, H_2
Acidobacteria, *Acidobacteria,* *Acidobacteriaceae*	5	2	2
Acidobacteria, *Holophagae*, *Holophagaceae*	27	-	2
Actinobacteria, *Actinobacteria*, *Iamiaceae*	2	-	-
Mycobacteriaceae	2	-	-
Solirubrobacteraceae	7	-	-
Streptomycetaceae	-	-	2
Thermomonosporaceae	-	-	2
Bacteroidetes, *Bacteroidia*, *Bacteroidaceae*	-	13	16
Marinilabiliaceae	-	-	2
Porphyromonadaceae	-	-	7
Bacteroidetes, *Sphingobacteriia*, *Chitinophagaceae*	-	2	-
Firmicutes, *Clostridia*, *Clostridiaceae*	2	6	7
Lachnospiraceae	-	9	-
Peptococcaceae	5	-	-
Ruminococcaceae	-	-	5
Firmicutes, *Negativicutes*, *Veillonellaceae*	-	13	21

Taxonomic level (phylum, class, family)	Relative abundance (%)[a]		
		After 21 days	
	Before incubation	Control	Formate, H_2
"*Nitrospira*", "*Nitrospira*",			
"*Nitrospiraceae*"	27	2	-
Proteobacteria, *Alphaproteobacteria*,			
Acetobacteraceae	-	2	2
Bradyrhizobiaceae	-	2	2
Methylocystaceae	-	-	2
Rhodospirillaceae	2	6	-
Roseiarcaceae	-	-	2
Proteobacteria, *Betaproteobacteria*,			
Neisseriaceae	-	2	2
Proteobacteria, *Deltaproteobacteria*,			
Desulfuromonadaceae	7	-	-
Family-level phylotype 1[b]	5	-	-
Proteobacteria, *Epsilonproteobacteria*,			
Campylobacteraceae	-	2	-
Proteobacteria, *Gammaproteobacteria*,			
Enterobacteriaceae	-	36	21
Verrucomicrobia, *Opitutae*,			
Opitutaceae	2	-	-
Others, Others[c],			
Family-level phylotype 2[b]	2	-	-
Family-level phylotype 3[b]	2	-	-
Family-level phylotype 4[b]	-	2	-

[a] The 16S rRNA gene sequences were based on an 87.5 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). Values are rounded to nearest whole number and thus might not sum up to 100 %. Legend: -, phylotype not detected.

[b] Sequences were considered to be a family-level phylotype without any cultured isolate if the 16S rRNA gene sequence was less than 87.5 % similar to the sequence of the closest related cultured species (Yarza *et al.* 2008).

[c] Listed family-level phylotypes do not necessarily belong to the same phylum or class.

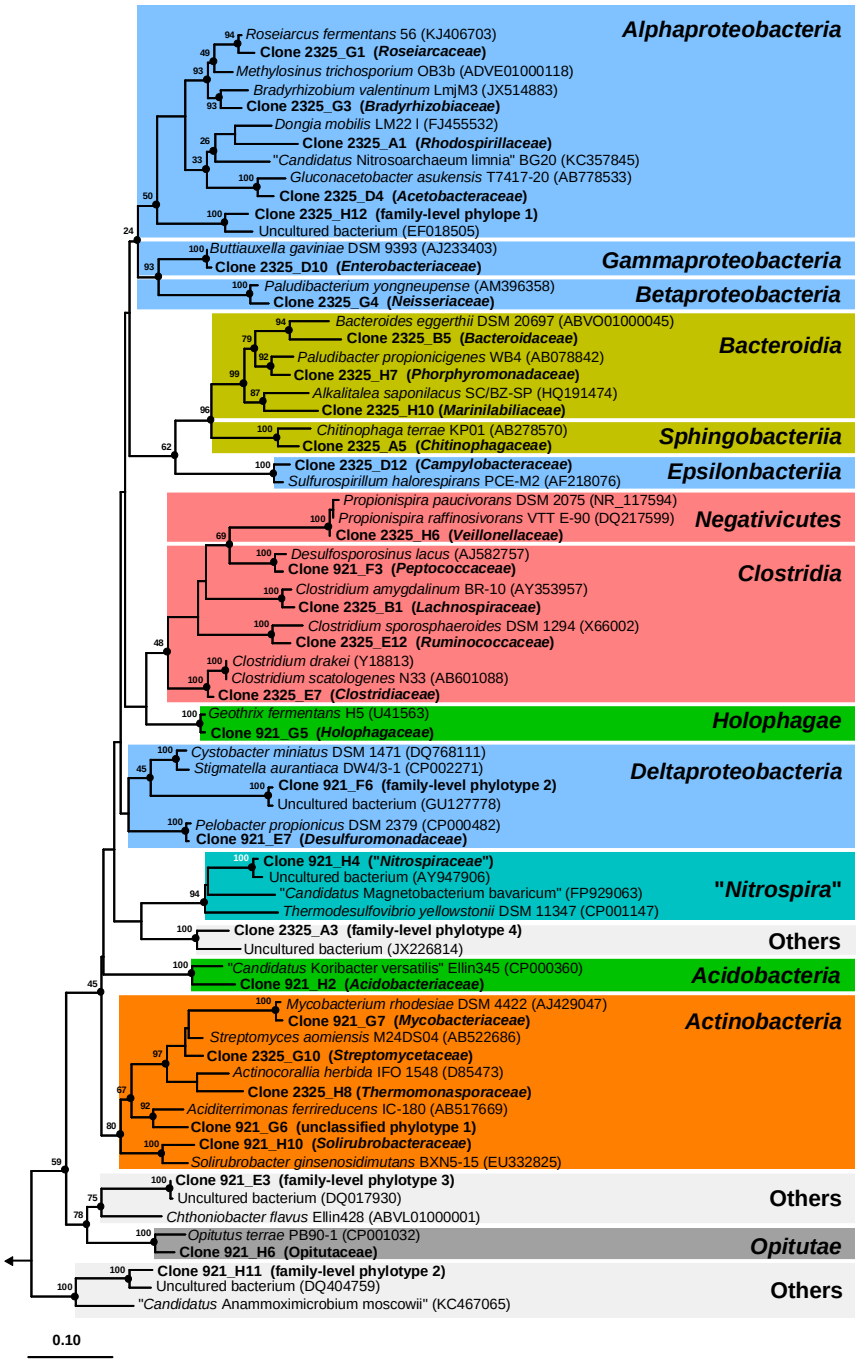


Figure 43: Phylogenic maximum parsimony tree of representative family-level 16S rRNA gene sequences from mire-derived roots and closely related sequences.

Sequences were obtained from mire-derived roots before and after 23 days of anoxic incubation. The 16S rRNA gene sequences were based on an 87.5 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). Accession numbers are indicated in brackets. Sequences correspond to nucleic acids 241-823 of the 16S rRNA gene sequence of *E. coli* (AB035923). Filled dots indicate congruent nodes in neighbor joining and maximum likelihood trees. Bootstrap values are from the maximum parsimony tree (1,000 resamplings) and are only displayed at nodes congruent in all three trees. The 16S rRNA gene sequence of *M. kandleri* (M59932) was used as outgroup. The bar indicates a 0.1 change per amino acid. Phyla were displayed color coded: blue, *Proteobacteria*; light green, *Bacteroidetes*; red, *Firmicutes*; dark green, *Acidobacteria*; light grey, taxa not affiliated to a phylum; orange, *Actinobacteria*; dark grey, *Verrucomicrobia*; pink, *Planctobacteria*.

5.4.7 Utilization of formate and other properties of FHL-containing isolates obtained from mire roots

Carex and *Molinia* roots from mire 2 had a very pronounced activity to form H_2 from formate by microorganisms that harbor enzymes such as FHL. To examine the type of microorganisms potentially associated with this activity, a mixture of *Carex* and *Molinia* roots from mire 2 was utilized to enrich FHL-containing taxa. The enrichment yielded two fermentative facultative aerobes, SB1 and SB2, that converted formate to H_2 and CO_2 at pH 5.0 (Figure 44).

SB1 stained Gram negative, formed single motile rods, and was oxidase and catalase negative. The 16S rRNA gene of SB1 was 99.6 % and 99.5 % similar to that of *Citrobacter freundii* and *Citrobacter braakii*, respectively, and the group 4 [NiFe]-hydrogenase gene sequence of SB1 was closely affiliated to that of *C. freundii* and *Citrobacter amalonaticus* (Figure 45), indicating that SB1 is a species of *Citrobacter*. A PCR signal with primers that target [FeFe]-hydrogenase genes was negative, indicating that SB1 does not contain a [FeFe]-hydrogenase.

Glucose, arabinose, citrate, and formate were utilized under both oxic and anoxic conditions at an *in situ*-relevant pH of 5.0 by SB1. Glucose, arabinose, citrate, and formate were completely oxidized at pH 5.0 after seven days when O_2 was available. Without O_2 available, supplemental substrates were not completely utilized at pH 5.0 (Table 37, Figure 44) but fermentation of supplemental glucose was enhanced nearly two-fold by supplemental formate given as co-substrate (Figure 44). Formate-supplemented cultures had a slightly elevated pH initially in comparison to cultures without formate (i.e., approximately 0.3 pH values higher). The difference in pH becomes more pronounced the longer the incubation runs. The utilization of glucose stops at a pH of 3.9 in glucose-supplemented cultures. In contrast, pH in glucose-formate-supplemented cultures were never lower than pH 4.7, indicating that the enhanced utilization of glucose in glucose-formate-supplemented cultures was due to a higher pH. The fermentation of glucose yielded ethanol, lactate, acetate, CO_2,

and H_2 as end products and showed the highest increase in optical density in comparison to the utilization of formate, citrate, and arabinose (Figure 44, Table 37).

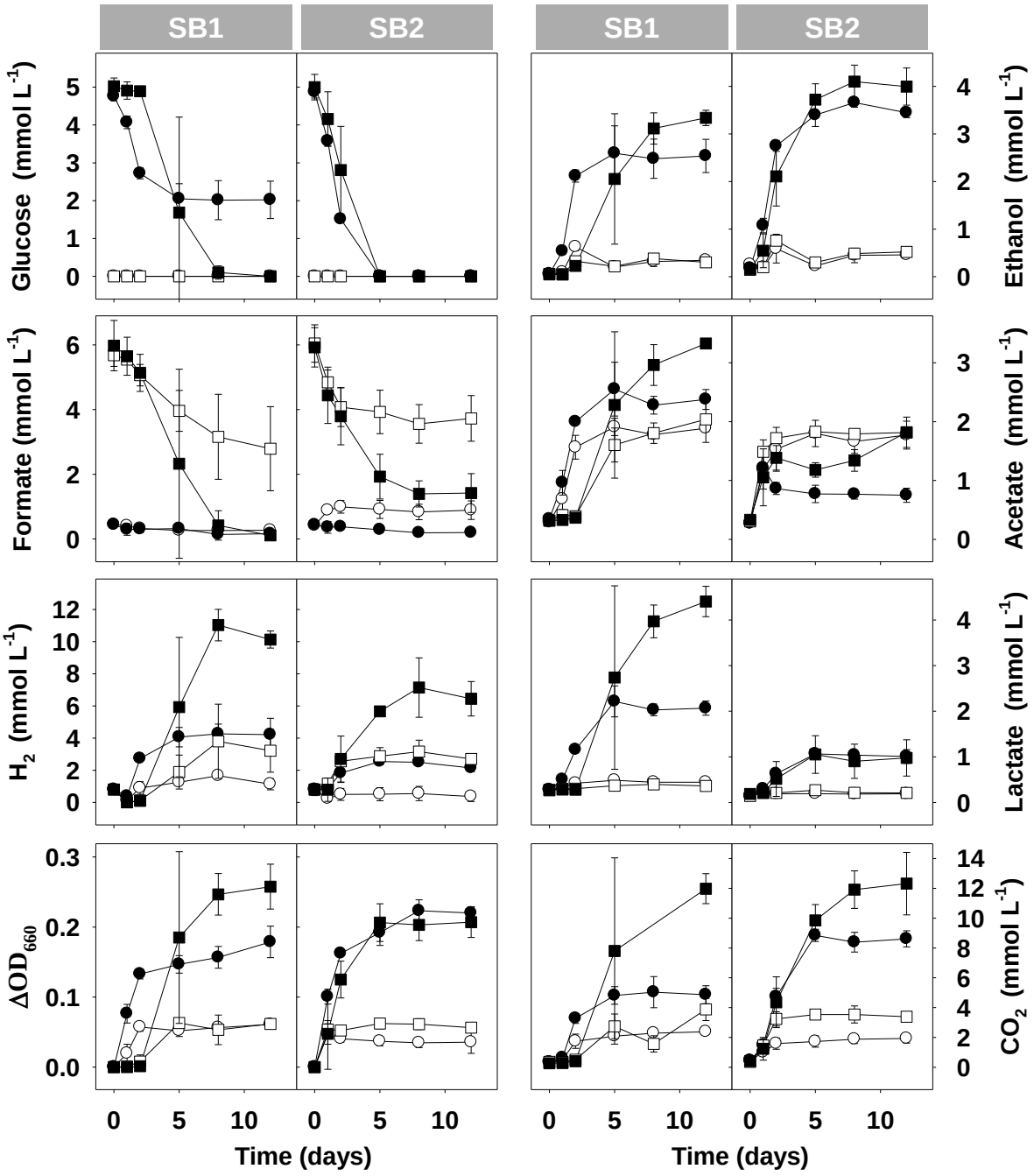


Figure 44: Effect of supplemental glucose and formate on the product profiles of isolates SB1 and SB2.

Citrobacter-related isolate SB1 and *Hafnia*-related isolate SB2 were obtained from a mixture of roots from mire 2. Isolates were incubated at 15 °C. Symbols: open circle, unsupplemented control; closed circle, glucose supplementation; open square, formate supplementation; closed square, supplementation of glucose and formate as co-substrates. Abbreviation: OD_{660}, optical density at 660 nm. Values are the mean of triplicate analysis and the error bars indicate the standard deviation. Figure was modified from Hunger *et al.* (2016).

Supplemental formate was converted in a 1:0.7 ratio to H_2 and in a 1:1 ratio to H_2 if glucose was supplemented as co-substrate under anoxic conditions (production of glucose-derived H_2 was subtracted), illustrating the FHL-activity of SB1 to produce H_2 from formate under different conditions. Arabinose-supplemented cultures showed a similar pH as the unsupplemented control before the incubation but nevertheless utilized arabinose weakly under anoxic conditions. Fermentation of arabinose yielded acetate, H_2, and CO_2 (Table 37). Anoxic utilization of citrate yielded succinate, acetate, and CO_2 as fermentation products.

Table 37: Effect of supplemental citrate and arabinose on product profiles of root-derived *Citrobacter*-related isolate SB1 under anoxic conditions.

	Concentrations of consumed substrates and released products after 14 days (mmol L^{-1}) and other parameters[a]		
	Unsupplemented	Citrate	Arabinose
Substrate consumed	n.a.	2.2 ± 0.6	0.4 ± 0.8
Amounts of products after 14 days (below)			
Succinate	-	3.4 ± 0.3	-
Formate	0.5 ± 0.1	0.1 ± 0.1	0.4 ± 0.1
Lactate	0.2 ± 0.1	-	0.2 ± 0.1
Acetate	1.4 ± 0.0	3.9 ± 0.3	1.9 ± 0.1
Ethanol	0.5 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
H_2	0.8 ± 0.1	0.7 ± 0.1	1.4 ± 0.1
CO_2	0.9 ± 0.1	3.6 ± 0.2	1.4 ± 0.2
pH before incubation	4.7 ± 0.0	6.3 ± 0.0	4.6 ± 0.0
pH after incubation	5.1 ± 0.1	6.4 ± 0.0	4.8 ± 0.0
ΔOD_{660}	40 ± 6	86 ± 7	51 ± 10

[a] *Citrobacter*-related isolate SB1 was obtained from a mixture of roots from mire 2. Approximately 5 mmol substrate L^{-1} were supplemented once at the beginning of the anoxic incubation. SB1 cultures were incubated at 15 °C and pH 5. Values are means of a triplicate analysis with standard deviation. A standard deviation of 0.0 mmol L^{-1} indicates a standard deviation that is smaller than 0.05 mmol L^{-1}. Legend: n.a., not applicable, -, no production or production lower than 0.05 mmol L^{-1}.

SB2 stained Gram negative, formed single motile rods, was oxidase negative, and catalase positive. The 16S rRNA gene of SB2 was 98.6 % and 99.6 % similar to that of *Hafnia alvei* and *Hafnia paralvei*, respectively, and the group 4 [NiFe]-hydrogenase gene sequence of SB2 was closely affiliated to that of *H. alvei* (Figure 45), indicating that SB2 is a species of *Hafnia*. A PCR signal with primers that target [FeFe]-hydrogenase genes was negative, indicating that SB2 does not contain a [FeFe]-hydrogenase.

Table 38: Effect of supplemental citrate and arabinose on product profiles of root-derived *Hafnia*-related isolate SB2 under anoxic conditions.

	Concentrations of consumed substrates and released products after 14 days (mmol L^{-1}) and other parameters[a]		
	Unsupplemented	Citrate	Arabinose
Substrate consumed	n.a.	5.0 ± 0.1	1.0 ± 0.6
Amounts of products after 14 days (below)			
Succinate	0.2 ± 0.0	4.4 ± 0.5	0.2 ± 0.2
Formate	0.5 ± 0.3	-	0.2 ± 0.4
Lactate	0.1 ± 0.0	-	0.2 ± 0.1
Acetate	1.5 ± 0.0	3.8 ± 0.3	2.3 ± 0.2
Ethanol	0.3 ± 0.0	0.1 ± 0.1	1.1 ± 0.3
H_2	0.5 ± 0.2	0.6 ± 0.1	2.1 ± 0.7
CO_2	0.8 ± 0.1	3.7 ± 0.5	1.6 ± 0.4
pH before incubation	5.0 ± 0.0	6.4 ± 0.0	4.9 ± 0.0
pH after incubation	5.0 ± 0.1	6.5 ± 0.0	4.7 ± 0.1
ΔOD_{660}	47 ± 7	75 ± 7	77 ± 5

[a] *Hafnia*-related isolate SB2 was obtained from a mixture of roots from mire 2. Approximately 5 mmol substrate L^{-1} were supplemented once at the beginning of the anoxic incubation. SB2 cultures were incubated at 15 °C and pH 5.0. Values are means of a triplicate analysis with standard deviation. A standard deviation of 0.0 mmol L^{-1} indicates a standard deviation that is smaller than 0.05 mmol L^{-1}. Legend: n.a., not applicable, -, not production or production lower than 0.05 mmol L^{-1}.

Glucose, arabinose, citrate, and formate were utilized under both oxic and anoxic conditions at an *in situ*-relevant pH of 5.0 by SB2. Glucose, arabinose, and formate were completely oxidized at pH 5.0 when O_2 was available and citrate was used weakly during

seven days of incubation. Without O_2 available, supplemental glucose, formate, and citrate were completely utilized at pH 5.0 but supplemental arabinose was used weakly (Table 38, Figure 44). In contrast to isolate SB1, isolate SB2 utilized glucose completely under anoxic conditions independent of (a) glucose being supplemented alone or as co-substrate with formate (Figure 44) and (b) an initially slightly elevated pH in formate-supplemented cultures in comparison to cultures without formate (i.e., approximately 0.3 pH values higher). The pH in glucose-supplemented and glucose-formate-supplemented cultures was never lower than pH 4.4 and pH 5.6, respectively. As has been shown with isolate SB1, formate utilization of isolate SB2 was enhanced when glucose was supplemented as co-substrate.

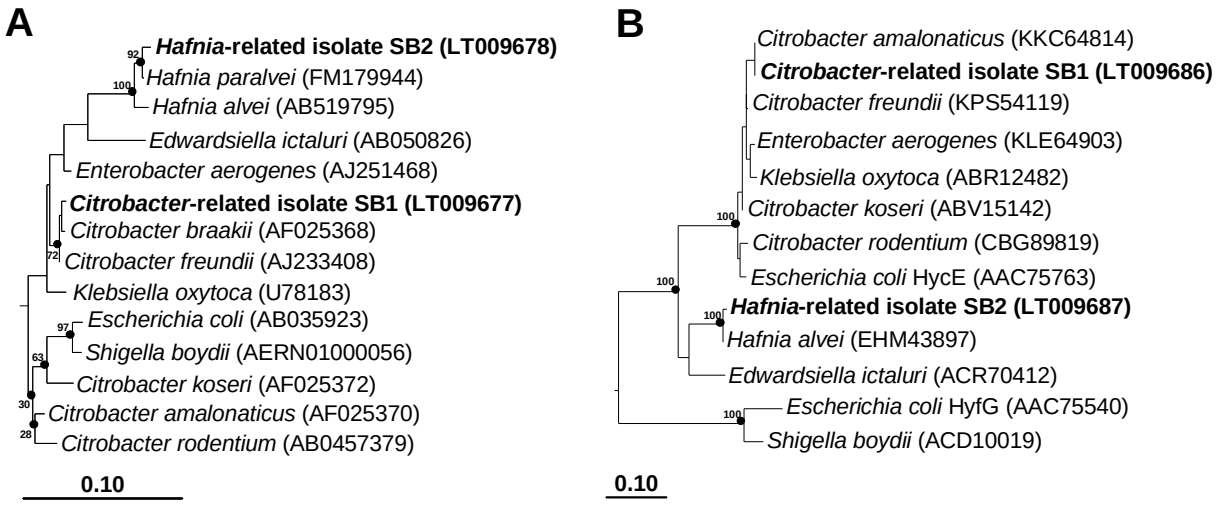


Figure 45: Phylogenic maximum parsimony trees of 16S rRNA gene sequences (A) and *in silico*-translated amino acid sequences derived from group 4 [NiFe]-hydrogenase genes (B) of isolates SB1 and SB2 and closely related sequences.

Accession numbers are indicated in brackets. 16S rRNA gene sequences correspond to residues 70-1439 of the 16S rRNA gene sequence of *E. coli* (AB035923). Hydrogenase amino acid sequences correspond to residues 246-528 of the *E. coli* hydrogenase 3 HycE protein (AAC75763). Filled dots indicate congruent nodes in the neighbor joining and the maximum likelihood tree. The 16S rRNA gene sequence (AB734660) and the hydrogenase amino acid sequence (ABC20475) of *M. thermoacetica* were used as outgroup in the respective trees. The bar indicates a 0.1 change per nucleic or amino acid. Bootstrap values are from the maximum parsimony tree (1,000 resamplings) and are only displayed at nodes congruent in all three trees. Figure was modified from Hunger *et al.* (2016).

The fermentation of glucose yielded ethanol, lactate, CO_2, and H_2 as end products. Supplemental formate was converted in a 1:1 ratio to H_2 independent of formate being supplemented alone or as co-substrate with glucose under anoxic conditions (production of

glucose-derived H_2 was subtracted), illustrating the FHL-activity of SB2 to produce H_2 from formate under different conditions. Arabinose was weakly utilized under anoxic conditions but resulted in a similar increase in optical density as utilization of citrate did (Table 38). Fermentation products from arabinose were acetate, ethanol, H_2, and CO_2. Anoxic utilization of citrate yielded succinate, acetate, and CO_2 as fermentation products.

5.4.8 Utilization of glucose and other properties of fermentative isolates obtained from mire roots

Two fermentative isolates, SB3 and SB4, were obtained from the same mixture of roots which *Citrobacter*-related isolate SB1 and Hafnia-related isolate SB2 derived from. Isolates SB3 and SB4 fermented glucose under anoxic and pH neutral conditions but could not grow or grew minimal under oxic or anoxic conditions at pH 5, indicating a lower tolerance to acidic conditions than isolates SB1 and SB2.

SB3 stained Gram negative, formed non-motile single rods, and was oxidase and catalase negative. The 16S rRNA gene of SB3 was 99 % similar to that of *Clostridium celerecrescens*, and *Clostridium sphenoides* (Figure 46), indicating that SB3 is a species of *Clostridium*. On a per liter basis, isolate SB3 converted approximately 5.0 mmol glucose to approximately 5.5 mmol ethanol, 3.1 mmol H_2, 2.7 mmol acetate, 1.3 mmol formate, and 0.2 mmol lactate under anoxic conditions (unsupplemented control was subtracted). CO_2 was used to prepare medium and was added as gaseous phase to tubes and thus was not monitored.

SB4 stained Gram positive, formed motile rods that occurred single in pairs or chains, and was oxidase and catalase negative. The 16S rRNA gene of SB4 was 99 % similar to that of *Carnobacterium maltaromaticum* (Figure 46), indicating that SB4 is a species of *Carnobacterium*. On a per liter basis, isolate SB4 converted approximately 5.2 mmol glucose to approximately 5.2 mmol formate, 3.1 mmol acetate, 2.9 mmol ethanol, and 0.9 mmol lactate under anoxic conditions (unsupplemented control was subtracted). H_2 was not detected. CO_2

was used to prepare medium and was added as gaseous phase to tubes and thus was not monitored.

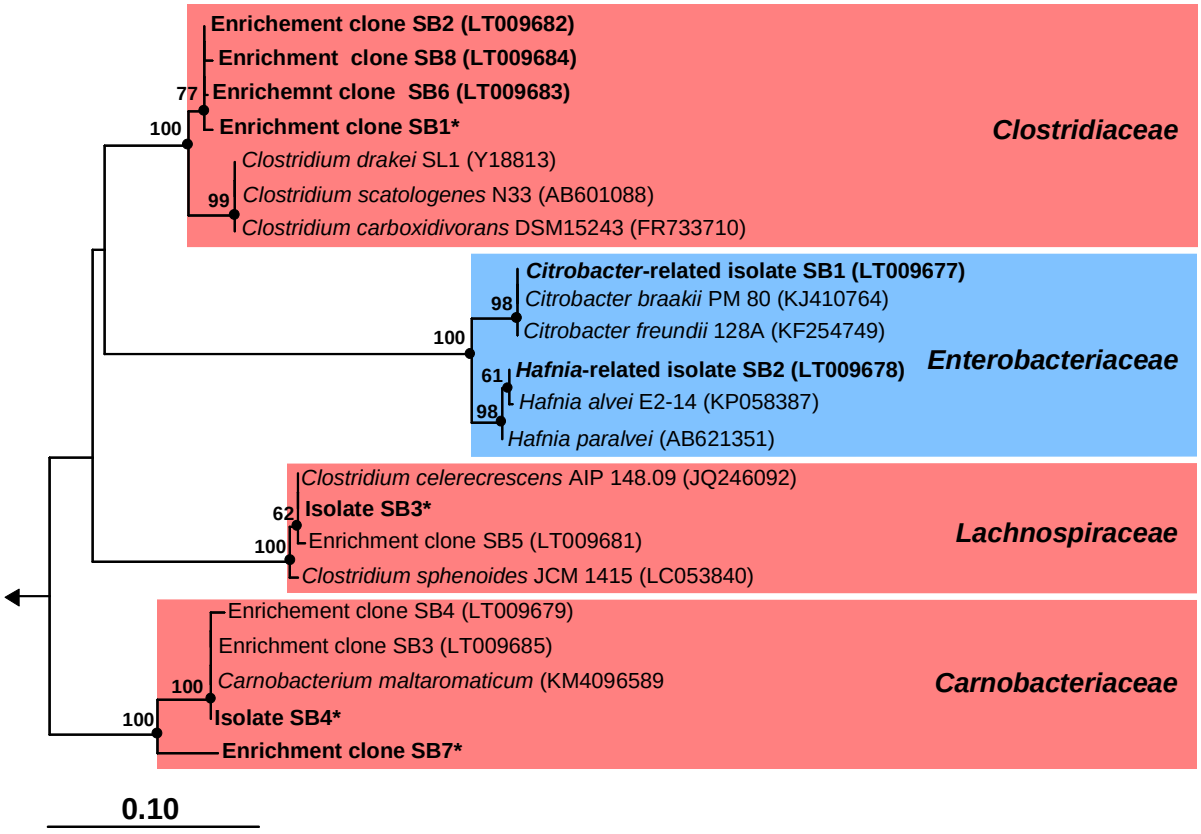


Figure 46: Phylogenic maximum parsimony tree of 16S rRNA gene sequences from mire-derived isolates, clone sequences from an acetogenic enrichment, and closely related sequences.

Accession numbers are indicated in brackets. Sequences correspond to residues 241-823 of the 16S rRNA gene sequence of *E. coli* (AB035923). Filled dots indicate congruent nodes in neighbor joining and maximum likelihood trees. The 16S rRNA gene sequence of *M. kandleri* (M59932) was used as outgroup. The bar indicates a 0.1 change per amino acid. Bootstrap values are from the maximum parsimony tree (1,000 resamplings) and are only displayed at nodes congruent in all three trees. Phyla were displayed color coded: blue, *Proteobacteria*; red, *Firmicutes*. Sequences marked with a star were not submitted to EMBL and thus have no accession number.

5.4.9 Acetogenic taxa associated with the root zone

The apparent H_2- and/or formate-driven synthesis of acetate and the detection of acetogen-affiliated 16S rRNA gene sequences (5.4.6, Figure 43) suggested that acetogens were associated with mire roots. However, attempts to isolate a representative clostridial acetogen from roots by numerous transfers in liquid, semi-solid, and solid medium were unsuccessful. The acetogenic enrichment produced acetate from H_2-CO_2, formate, lactate,

and CO during the enrichment process. The final acetogenic enrichment (designated FH) derived from the same mixture of roots where SB1, SB2, SB3, and SB4 derived from and contained one acetogen that was closely related to *C. drakei*, *C. scatologenes*, *C. carboxidivorans*, and *C. magnum*, and two fermenters, one closely related to *Clostridium celerecrescens* and isolate SB3, and the other closely related to *C. maltaromaticum* and isolate SB4 (Figure 46). Enrichment FH produced acetate from formate. Despite the vitamins and yeast extract-derived nutrients provided in the enrichment medium, the acetogenic activity of the enrichment was lost after a few transfers in medium that lacked autoclaved roots or root extract. Thus, unknown plant-derived nutrients appeared to be important for maintaining the enriched clostridial acetogen.

5.5 Anaerobic processes in gut contents of the CH_4-emitting earthworm *E. eugeniae*

5.5.1 Diversity of *mcrA*/*mrtA* genes and transcripts associated with gut contents

It has recently been discovered that earthworms such as *E. eugeniae* emit various amounts of CH_4 *in vivo* and can be as high as 41 nmol CH_4 per g[earthworm$_{FW}$]$^{-1}$ in 5 hours (Depkat-Jakob *et al.* 2012). Based on this observation, the gut contents of *E. eugeniae* was analyzed for the presence of *mcrA*/*mrtA* genes and transcripts to resolve methanogenic taxa potentially associated with the emission of CH_4.

In total, 367 *mcrA*/*mrtA* sequences from gut contents of *E. eugeniae* and from the substrate used to raise *E. eugeniae* on (i.e., composted cow manure) were obtained and clustered into 12 species-level phylotypes, eight family-level *mcrA* phylotypes and one family-level *mrtA* phylotype (Table 39, Figure 48); including *Methanobacteriaceae*, *Methanocellaceae*, *Methanomicrobiaceae*, *Methanoregulaceae*, *"Methanosaetaceae*", *Methanosarcinaceae*, *Methanospirillaceae*, and one family-level phylotype without any cultured isolates (Figure 48).

Table 39: Coverage of clone libraries, number of *mcrA*/*mrtA* sequences and transcripts and number of species-level *mcrA*/*mrtA* phylotypes obtained from gut contents of *E. eugeniae* and substrate.

Target	Clone libraries	No. of sequences	No. of phylotypes	Coverage
mcrA/*mrtA* gene	Gut contents	94	6	>99
	Substrate[a]	87	8	99
mcrA transcript	Gut contents	94	5	99
	Substrate[a]	92	7	98
	Total:	367	12	>99

[a] Composted cow manure was used as substrate to raise earthworms on.

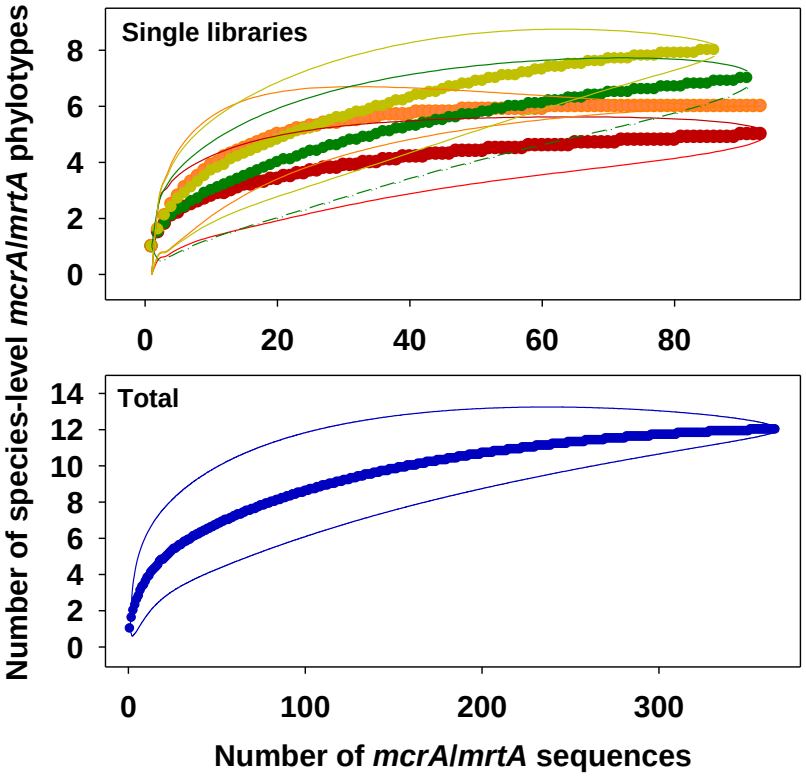


Figure 47: Rarefaction analysis of species-level *mcrA*/*mrtA* phylotypes obtained from gut contents of *E. eugeniae* and substrate.

Samples were taken from gut contents of *E. eugeniae* and the substrate used to raise the earthworms on (i.e., composted cow manure). *mcrA*/*mrtA* sequences were *in silico* translated into amino acids and based on an 85.7 % similarity cut-off clustered into species-level phylotypes (5.1.2). 95 % confidence intervals are shown. Curves were calculated according to the Hurlbert rarefaction (Hurlbert 1971). Symbols: red, *mcrA* transcript sequences from gut contents; orange, *mcrA*/*mrtA* sequences from gut contents; green, *mcrA* transcript sequences from substrate; yellow, *mcrA*/*mrtA* sequences from substrate. Figure was modified from Depkat-Jakob *et al.* (2012).

Rarefaction curves and coverage indicated that sampling was sufficient for species-level clustering of *mcrA*/*mrtA* phylotypes (Table 39, Figure 47). The diversity of species-level *mcrA*/*mrtA* phylotypes and species-level *mcrA*/*mrtA* transcript phylotypes was similar in gut contents and substrate (Table 39, Figure 47).

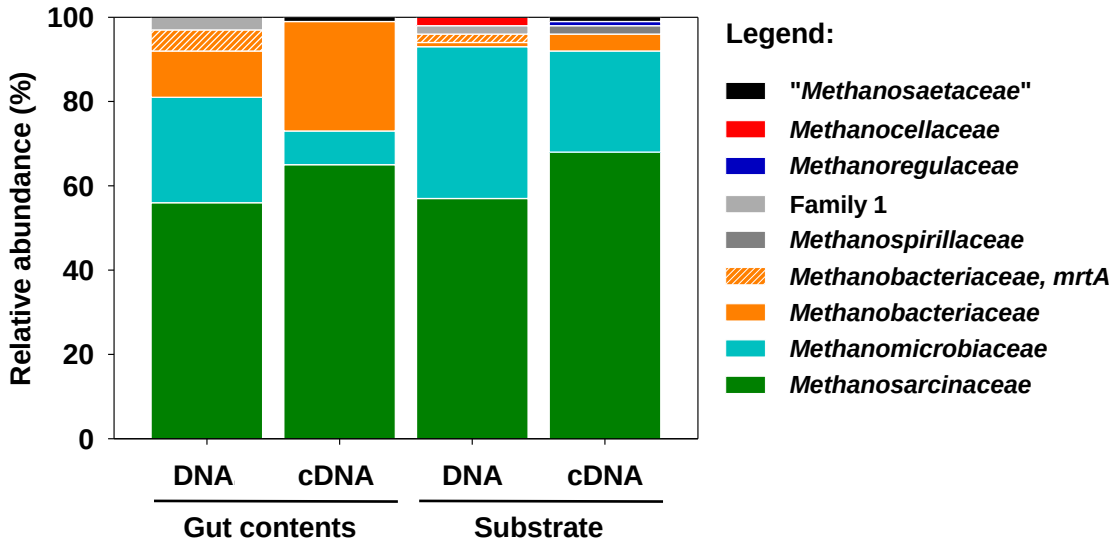


Figure 48: Relative abundance of family-level *mcrA*/*mrtA* phylotypes obtained from gut contents of *E. eugeniae* and substrate.

Samples were taken from gut contents of *E. eugeniae* and the substrate used to raise the earthworms on (i.e., composted cow manure). Family 1 is a family-level *mcrA* phylotype without cultured isolates. *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net).

The *Methanosarcina mazei*-affiliated phylotype (*Methanosarcinaceae*) was the most abundant species-level *mcrA* phylotype in gut contents of *E. eugeniae* and substrate in gene and transcript level analysis and accounted for 55-67 %, suggesting that most of the present *Methanosarcinaceae*-affiliated taxa were active in gut contents and substrate (Figure 48, Figure 49). *Methanomicrobiaceae* had a relative abundance of 25 % and 26 % on gene level in gut contents and substrate, respectively, and 8 % and 24 % on transcript level in gut contents and substrate, respectively, indicating that a minor part of *Methanomicrobiaceae*-affiliated taxa was active in gut contents. *Methanobacteriaceae* had a higher relative abundance on transcript level than on gene level and also a higher relative abundance in gut contents than in the substrate, indicating that *Methanobacteriaceae*-affiliated taxa were activated in gut contents. Species-level *mcrA* phylotype PLT11 was most closely related to

Methanobacterium formicicum (94 % amino acid sequence similarity, EF465108) and showed the highest relative abundance of *Methanobacteriaceae*-affiliated species-level phylotypes (Figure 49). *Methanosaetaceae*-, *Methanospirillaceae*- and *Methanoregulaceae*-affiliated phylotypes had very low relative abundances and were only detected at the transcript level.

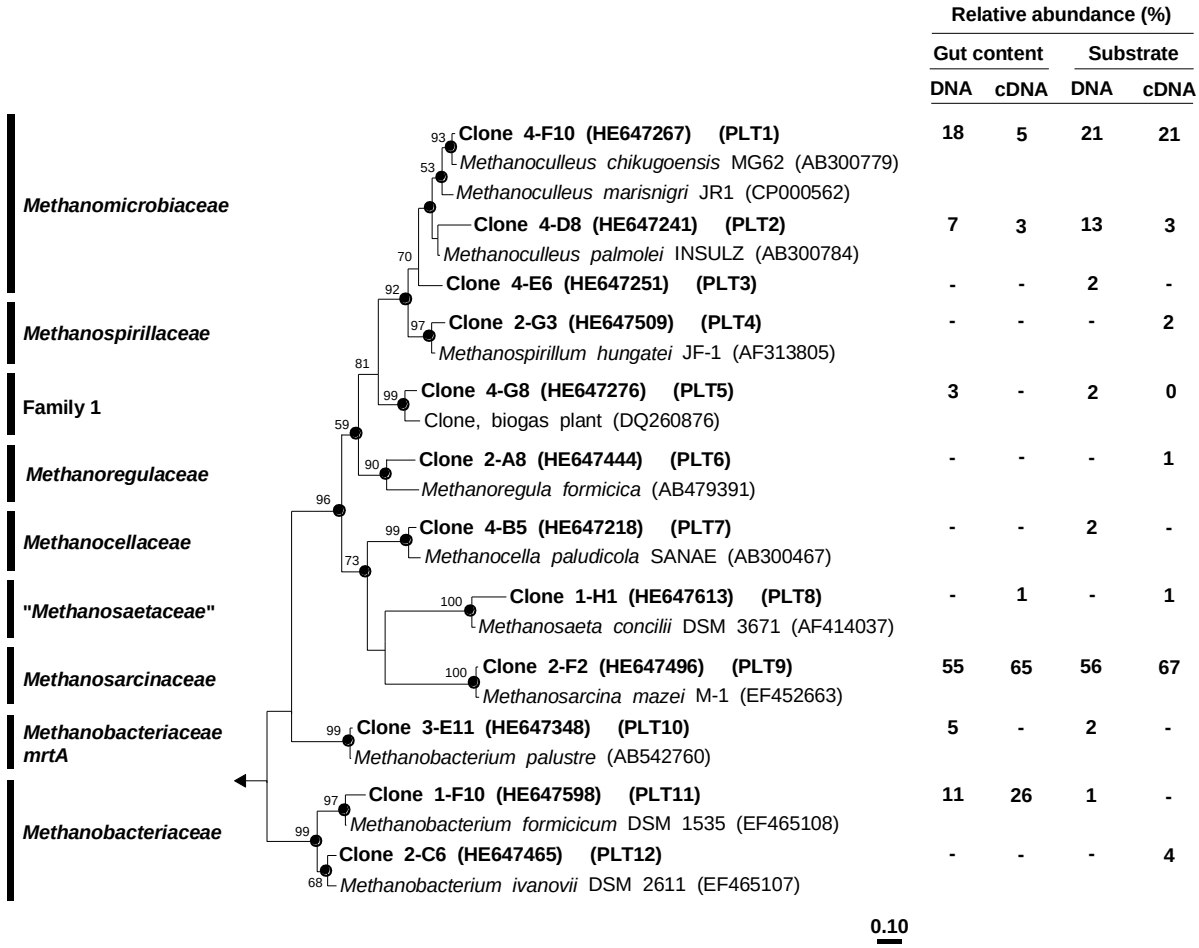


Figure 49: Phylogenic neighbor-joining tree of (a) representative *mcrA*/*mrtA*-encoded amino acid sequences retrieved from gut contents of *E. eugeniae* or substrate and (b) reference sequences.

Sequences were obtained from gut contents of *E. eugeniae* and the substrate used to raise the earthworms on (i.e., composted cow manure). *mcrA*/*mrtA* sequences were *in silico* translated into amino acids and based on an 85.7 % similarity cut-off clustered into species-level phylotypes (5.1.2). *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net). Accession numbers are indicated in brackets. Sequences correspond to residues 98-227 of the *mcrA*-encoded amino acid sequence of *M. paludicola* (AB300467). Dots at nodes indicate the confirmation of tree topology by all maximum likelihood and maximum parsimony calculations with the same data set. *M. kandleri* (AE009439) was used as outgroup. The bar indicates a 0.1 estimated change per amino acid. Values next to the branches represent the percentages of replicate trees (>50 %) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps). Legend: PLT, phylotype; -, not detected. Values are rounded to nearest whole number. Figure was modified from Depkat-Jakob *et al.* (2012).

Species-level phylotype PLT5 (family-level phylotype 1) was without cultured isolate and shares 72-84 % *mcrA*-encoded amino acid similarity to its next cultured relatives *Methanosphaerula palustris* (EU296536, 83-84 % similarity), *Methanoculleus palmolei* (AB300784, 79-84 % similarity), and *Methanoregula formicica* (AB479391, 72-77 % similarity).

5.5.2 Properties of a methanogenic enrichment derived from gut contents

Sequence analysis of *mcrA*/*mrtA* gene and transcript analysis of gut contents derived from *E. eugeniae* indicated the presence of different methanogens in the gut of *E. eugeniae*. Methanogens were enriched from gut contents of *E. eugeniae*. to analyze potential properties of those methanogens in the gut.

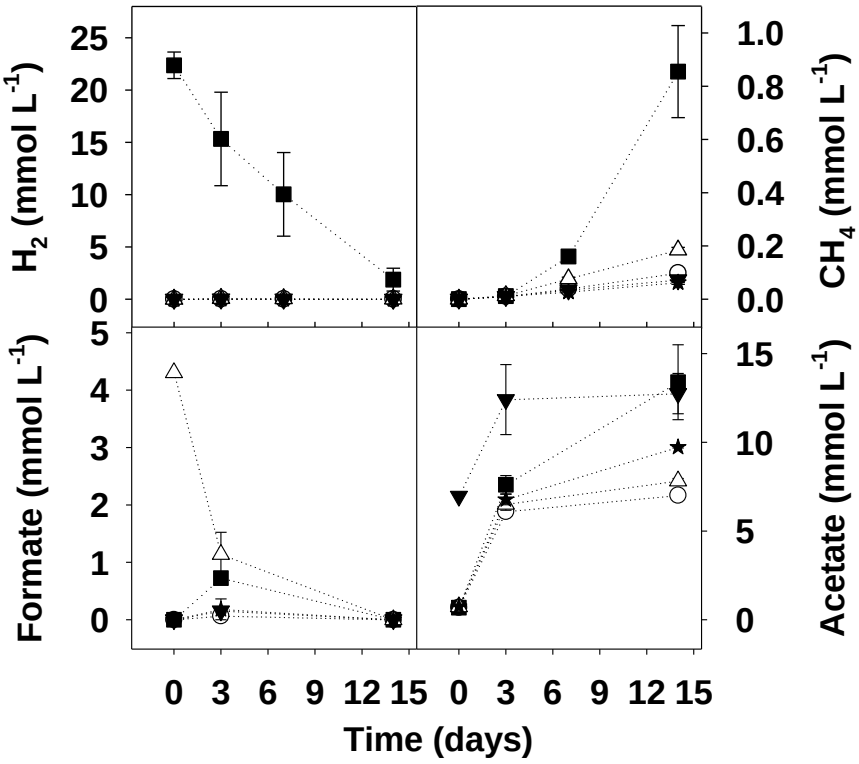


Figure 50: Effect of supplemental H_2, formate, acetate, and methanol on product profiles of a methanogenic enrichment.

Incubation was performed at 25 °C. Methanol concentrations could not be determined and consumption of methanol is unresolved. Symbols: open circle, unsupplemented control; closed squares, H_2 supplementation; open pyramid, formate supplementation; closed stars, methanol supplementation; closed triangle, acetate supplementation. Values are means of triplicate analysis and the error bars indicate the standard deviation.

The methanogenic enrichment culture was supplemented with H_2, formate, acetate, or methanol and incubated for 14 days under anoxic conditions. The production of CH_4 was highest with supplemental H_2 followed by supplemental formate (Figure 50). Supplemental acetate and methanol did not stimulate the production of CH_4. Surprisingly, supplemental H_2 and methanol stimulated the production of acetate, suggesting the formation of acetate by acetogenesis. H_2-derived production of acetate exceeded the production of H_2-derived CH_4 considerably.

Table 40: Relative abundance of species-level *mcrA*/*mrtA* phylotypes from a methanogenic enrichment obtained from gut contents of *E. eugeniae*.[a]

Relative abundance (%)	Representative sequence	Closest cultured sequence	Similarity (%)
39	C01 (LK936474)	*Methanobacterium ivanovii mrtA* (EF465104)	97-100
5	B02 (LK936469)	*Methanobacterium formicicum mrtA* (EF465103)	98-99
5	B05 (LK936472)	*Methanobacterium formicicum mcrA* (EF465108)	100
15	C06 (LK936477)	*Methanobacterium* sp. *mcrA* (DQ677519)	94
2	A03 (LK936464)	*Methanobacterium subterraneum mcrA* (BAI67103)	98
34	D06 (LK936483)	*Methanobacterium ivanovii mcrA* (EF465107)	97-98

[a] *McrA*/*mrtA* sequences were *in silico* translated into amino acids and based on an 85.7 % similarity cut-off clustered into species-level phylotypes (5.1.2). Values for similarity are based on *mcrA*-encoded amino acid sequences. Accession numbers are indicated in brackets.

In total, 41 *mcrA*/*mrtA* sequences were obtained from the H_2-supplemented methanogenic enrichment. Those sequences clustered into four species-level *mcrA* phylotypes and two species-level *mrtA* phylotypes affiliated with *Methanobacteriaceae* (Table 40). Sequences affiliated with *Methanobacterium ivanovii* were most abundant in the H_2-supplemented methanogenic enrichment.

5.5.3 Effect of supplemental [^{13}C]glucose and H_2 on fermentation and acetogenesis in anoxic slurries with gut contents

The consumption of H_2 and the production of a considerable amount of acetate in the methanogenic enrichment (Figure 50), indicated the presence of hitherto unknown acetogens in gut contents of *E. eugeniae*. Attempts were made to identify acetogens in mire ‘soil’ by DNA

SIP with [^{13}C]formate (5.3, Hunger *et al.* 2011a). Unfortunately, attempts remained mostly unsuccessful, potentially because of (a) labeled taxa were not identified as acetogens (i.e., next related isolates affiliated with labeled taxa were no acetogens and thus labeled taxa were not identified as an acetogen) or (b) mire-derived acetogens dissimilated [^{13}C]formate and assimilated endogenous ^{12}C-compounds and thus were not labeled. In this regard, the Gibbs free energy under standard conditions for formate-dependent acetogenesis is -99 kJ mol^{-1} whereby the Gibbs free energy under standard conditions for glucose-dependent acetogenesis is about seven times higher (i.e., -687 kJ mol^{-1}) (own calculations), illustrating that dissimilation of glucose is thermodynamically more favorable than that of formate and might lead to an enhanced assimilation of glucose. Glucose can be detected in millimolar concentrations in gut contents of earthworms and successfully served as model saccharide for analyses of microbially mediated anaerobic processes in the gut contents before (Wüst *el al.* 2011, Schulz *et al.* 2015). Thus, an RNA SIP analysis was conducted with [^{13}C]glucose in the attempt to identify active acetogens. H_2 and $^{12}CO_2$ were supplemented as co-substrates to (a) minimize labeling of taxa by fermentation-derived $^{13}CO_2$ during incubation and (b) additionally stimulate acetogens.

Unsupplemented slurries with gut contents of *E. eugeniae* produced mostly acetate followed by CO_2, propionate, H_2, butyrate and traces of formate and CH_4 (Figure 51, data for butyrate not shown). Butyrate accumulated equally in all slurries to a final concentration of 10 µmol g[gut content$_{FW}$]$^{-1}$ (data not shown). Traces of lactate were detected before the incubation and were consumed within the first two days (data not shown). Succinate was detected in all slurries before the incubation but was only consumed in unsupplemented slurries, most likely due to the slightly more neutral pH of the unsupplemented controls (i.e., pH 6.9 in unsupplemented controls versus pH 6.3 in [^{13}C]glucose- and [^{13}C]glucose-H_2-supplemented slurries after seven days of incubation).

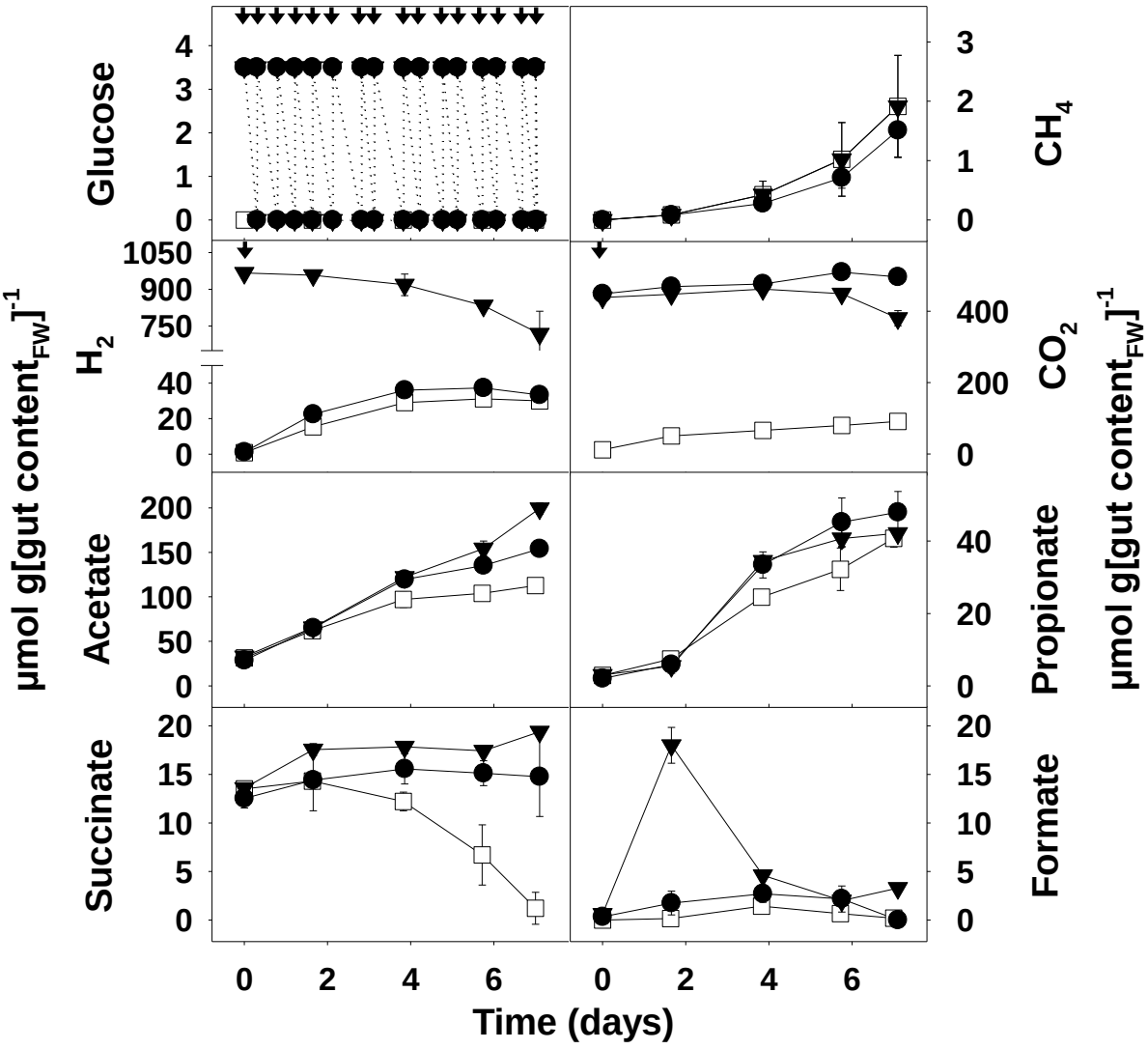


Figure 51: Effect of supplemental [^{13}C]glucose and H_2 on product profiles of gut contents from the earthworm *E. eugeniae*.

Incubation was performed at 25 °C. Symbols: open squares, unsupplemented controls; closed circles, [^{13}C]glucose-supplemented slurries; closed triangle, [^{13}C]glucose- and H_2-supplemented slurries. Arrows indicate time point of supplementation. Slurries that were supplemented with [^{13}C]glucose were also supplemented with $^{12}CO_2$ once at the beginning of incubation to dilute fermentation-derived $^{13}CO_2$ during incubation. Values are means of triplicate analysis. Error bars indicate the standard deviation.

Supplemental glucose was consumed without delay in all glucose-supplemented slurries with gut contents and stimulated predominantly the production of acetate (Figure 50, Table 42). Succinate was detected before the supplementation of glucose and glucose consumption enhanced succinate concentrations only slightly. Other typical fermentation products besides succinate, such as H_2 and propionate, were also enhanced slightly in

glucose-supplemented slurries in comparison to unsupplemented controls. Formate was detected transiently in glucose-H_2-supplemented slurries and might have also been produced transiently in glucose-supplemented slurries without supplemental H_2, indicating that some fermentation products were produced and quickly consumed by gut-derived microorganisms. Considering the transient appearance of formate (Figure 51) and the high ^{13}C-enrichment of slightly stimulated fermentation products such as succinate and propionate (17-29 at %, Table 41), it seems that production and consumption of glucose-derived fermentation products can be tightly coupled. The recovery of glucose-derived carbon and reductant of approximately 50 % indicated that a part of glucose-derived carbon and reductant might have been assimilated and/or was not detected. Production of CH_4 was not stimulated in slurries with supplemental glucose (Figure 51), but nevertheless was enriched with 13 at % of ^{13}C (Table 41), indicating that CH_4 partially derived from ^{13}C-enriched fermentation products.

Table 41: Enrichment of ^{13}C in products of anoxic slurries after seven days of incubation.

	^{13}C-content after 7 days (at %)[a]					
Treatment	Acetate	Succinate	Propionate	Butyrate	CH_4	CO_2
Unsupplemented control	1.2 ± 0.0	1.1[b]	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
[^{13}C]glucose, CO_2	42 ± 1	25 ± 6	20 ± 2	20 ± 1	13 ± 1	18 ± 0.0
[^{13}C]glucose, H_2, CO_2	36 ± 1	29 ± 1	17 ± 1	19 ± 1	13 ± 1	19 ± 1

[a] Values are means of triplicate analysis with standard deviation. A standard deviation of 0.0 ^{13}C at % indicates a standard deviation that is smaller than 0.05 ^{13}C at %.
[b] Succinate in the unsupplemented control was only detected in one replicate and thus the given value represents the succinate concentration in one of three replicates.

H_2 was supplemented as co-substrate to half of the glucose-supplemented slurries. Supplemental H_2 was consumed after a short lag phase of two days and stimulated the production of acetate in comparison to glucose-supplemented slurries lacking supplemental H_2 (Figure 51, Table 42). This additional production of acetate occurred concomitant with the consumption of H_2, and the ratio of consumed H_2 (220 µmol H_2 g[gut content$_{FW}$]$^{-1}$) to produced

acetate (46 µmol acetate g[gut $content_{FW}$]$^{-1}$) approximated 4:0.84 which is indicative for H_2-dependent acetogenesis. Acetate was less enriched in ^{13}C in glucose-supplemented slurries with supplemental H_2 (i.e., 36 at %) than in slurries lacking supplemental H_2 (i.e., 42 at %) (Table 41), indicating that acetate in slurries with supplemental H_2 was partially produced from ^{12}C-compounds such as $^{12}CO_2$ by hydrogenotrophic acetogenesis.

Table 42: Recovery of reductant and carbon from supplemental glucose, CO_2, and H_2 in anoxic slurries with gut contents of *E. eugeniae*.

	Carbon recovered from glucose and CO_2 (%)[a]		Reductant recovered from glucose and H_2 (%)[a]	
Products	With H_2	Without H_2	With H_2	Without H_2
Acetate	36	24	36	26
Succinate	15	16	13	15
Propionate	1	7	1	8
Formate	1	0	0.3	0
Total:	53	47	50	49

[a] Recovery was calculated based on process data at the beginning of incubation and after seven days of glucose supplementation (Figure 51). Values are rounded to nearest whole number.

5.5.4 Bioenergetics of H_2- and glucose-dependent acetogenesis

H_2- and glucose-dependent acetogenesis was thermodynamically feasible in all anoxic slurries with gut contents of *E. eugeniae* during the incubation (Figure 52). The Gibbs free energy of glucose-dependent acetogenesis was more negative than that of H_2-dependent acetogenesis.

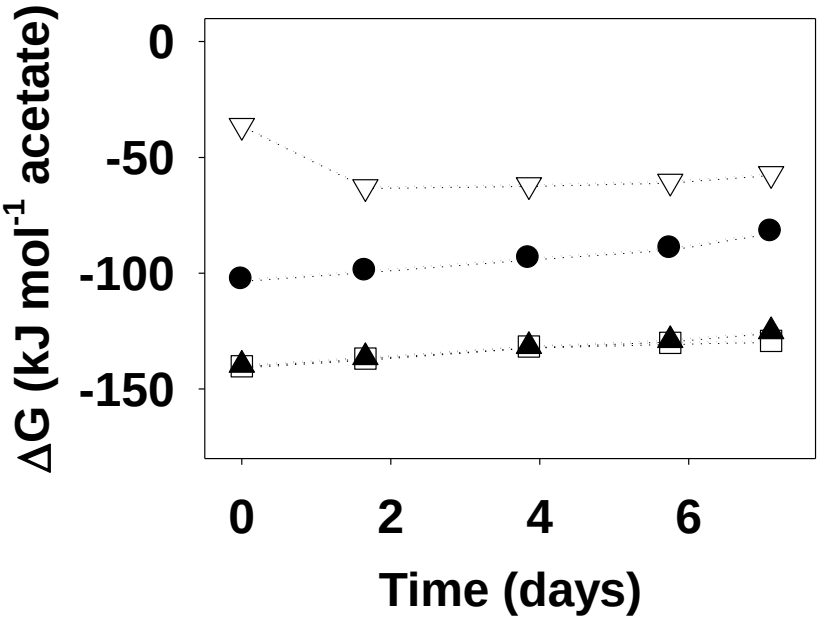


Figure 52: Estimated Gibbs free energy (ΔG) of H_2- and glucose-dependent acetogenesis in anoxic slurries with gut contents of *E. eugeniae*.

Process data can be found in Figure 51. Legend: open squares, glucose-dependent acetogenesis in [^{13}C]glucose-supplemented slurries; closed pyramid, glucose-dependent acetogenesis in [^{13}C]glucose- and H_2-supplemented slurries; closed circles, H_2-dependent acetogenesis in [^{13}C]glucose- and H_2-supplemented slurries; open triangle, H_2-dependent acetogenesis in [^{13}C]glucose-supplemented slurries. Values are means of triplicate analysis. Standard deviation was plotted but was too little to appear as error bars. The plot shows the change of ΔG values per acetate produced. Consequently, ΔG values for glucose-dependent acetogenesis is three times higher than ΔG values for H_2-dependent acetogenesis considering the whole reaction.

5.5.5 Diversity of bacterial 16S rRNA phylotypes in [^{13}C]glucose-supplemented slurries

After the isopycnic centrifugation of RNA in a cesium trifluoroacetate gradient and subsequent fractionation (4.10.7), heavy RNA from fractions three and four were pooled, and RNA from light fractions eight and nine were pooled, and used for the molecular analysis of bacterial 16S rRNA.

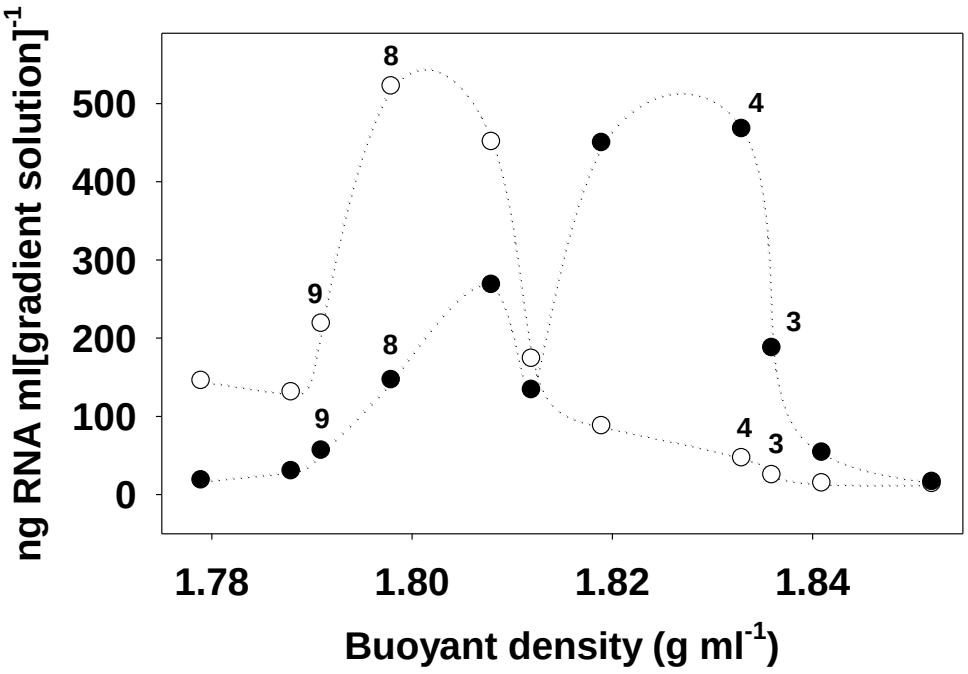


Figure 53: Distribution of RNA obtained from anoxic slurries with gut contents of *E. eugeniae* in cesium trifluoroacetate gradient.

Process data can be found in Figure 51. Legend: open circles, RNA from gut contents before the incubation; closed circles, RNA from [^{13}C]glucose- and H_2-supplemented slurries after seven days of incubation. Numbers indicate fractions that were used for molecular analysis.

In total, 601 bacterial 16S rRNA sequences were obtained that clustered into 33 family-level phylotypes and four phylum-level phylotypes (Table 43) including *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Planctobacteria* (Table 44). Rarefaction curves and coverage indicated that sampling was sufficient for family-level clustering of 16S rRNA phylotypes (Table 43, Figure 54). The diversity of family-level 16S rRNA gene phylotypes was the highest in light fractions of [^{13}C]glucose-supplemented slurries lacking supplemental H_2 and the lowest in heavy fractions of [^{13}C]glucose-supplemented slurries independent of supplemental H_2, indicating the occurrence of specifically enriched phylotypes in heavy fractions.

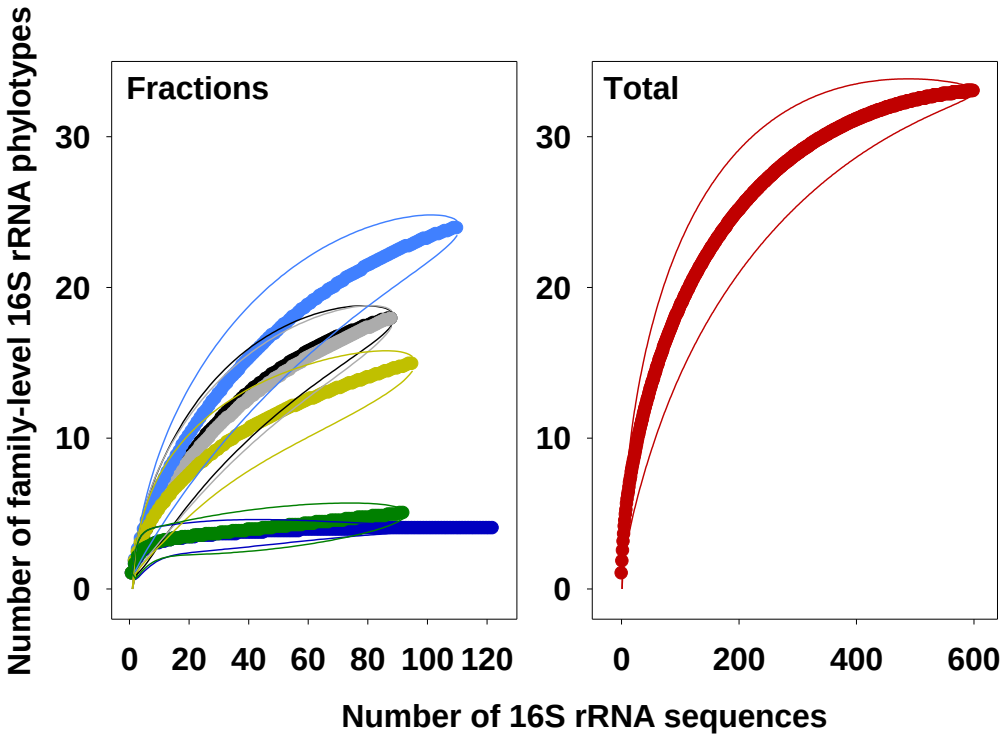


Figure 54: Rarefaction analysis of family-level 16S rRNA phylotypes obtained from gut contents of *E. eugeniae*.

Sequences derived from slurries with gut contents of *E. eugeniae* before and after seven days of anoxic incubation (Figure 48). Assignment of 16S rRNA gene sequences to family-level phylotypes was based on an 87.5 % similarity cut-off (Yarza *et al.* 2008). A 95 % confidence interval is shown. 16S rRNA sequences derived from light and heavy fractions of cesium trifluoroacetate gradients after isopycnic centrifugation. Color code: black, light fraction before incubation; light grey, light fraction of unsupplemented control; light blue, light fraction of [^{13}C]glucose-supplementation; dark blue, heavy fraction of [^{13}C]glucose-supplementation; light green, light fraction of [^{13}C]glucose-H_2-supplementation; dark green, heavy fraction of [^{13}C]glucose-H_2-supplementation; red, total number of sequences.

Family-level 16S rRNA phylotypes affiliated with *Enterobacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* showed the highest relative abundance (Table 44). *Peptostreptococcaceae* and *Hyphomicrobiaceae* were the most abundant taxa in light fractions derived from slurries before the incubation, indicating that affiliated microorganisms in gut contents were not stimulated by the experimental conditions. Species-level 16S rRNA phylotypes PLT1 and PLT2 (*Peptostreptococcaceae*) were affiliated with *Clostridium bifermentans* (98 % sequence similarity, X75906) and *Eubacterium tenue* (98 % sequence similarity, DQ445860), and accounted for 17 % and 26 % in light fractions derived from gut contents before supplementation of [^{13}C]glucose (Figure 55), respectively. One species-level 16S rRNA phylotype PLT4 (*Peptostreptococcaceae*) was most closely

related to the acetogen *Terrisporobacter glycolicus* RD-1 (99 % sequence similarity, AJ291746), but was only detected in light fractions derived (a) from unsupplemented slurries after the incubation and (b) from [^{13}C]glucose-supplemented slurries with supplemental H_2, indicating that affiliated microorganisms did not assimilate glucose-derived ^{13}C.

Table 43: Coverage of clone libraries, number of 16S rRNA sequences, and number of family-level 16S rRNA phylotypes obtained from anoxic slurries with gut contents of *E. eugeniae*.

Target	Clone libraries	No. of sequences	No. of phylotypes	Coverage
16S rRNA	Before incubation L[a]	89	18	94
	Control L[a]	89	18	92
	[^{13}C]glucose L[a]	111	23	95
	[^{13}C]glucose H[b]	123	4	>99
	[^{13}C]glucose-H_2 L[a]	96	15	94
	[^{13}C]glucose-H_2 H[b]	93	5	98
	Total:	601	33	>99

[a] Sequences derived from light fractions eight and nine.
[b] Sequences derived from heavy fractions three and four.

Enterobacteriaceae and *Lachnospiraceae* had a higher relative abundance in heavy fractions than in light fractions derived from slurries after [^{13}C]glucose-supplementation (Table 44), indicating that affiliated microorganisms assimilated glucose-derived ^{13}C. Species-level 16S rRNA phylotypes PLT20 and PLT21 (*Enterobacteriaceae*) were affiliated with *Citrobacter murliniae* (99 % sequence similarity, AF025369) and *Erwinia persicina* (98 % sequence similarity, AJ001190), and accounted for 16 % and 14 % in heavy fractions derived from [^{13}C]glucose-supplemented slurries with supplemental H_2 (Figure 55), respectively. The species-level 16S rRNA phylotype PLT7 (*Lachnospiraceae*) affiliated with *Robinsoniella peoriensis* (99 % sequence similarity, AF445198) and accounted for 51 % and 46 % in heavy fractions derived from [^{13}C]glucose-supplemented slurries lacking H_2 and slurries with supplemental H_2, respectively, and was more abundant in heavy fractions than in corresponding light fractions, indicating that affiliated microorganisms assimilated glucose-derived ^{13}C.

Table 44: Relative abundance of family-level 16S rRNA phylotypes from gut contents of *E. eugeniae* and phylogenic affiliations.

	Relative abundance of 16S rRNA sequences (%)[a]					
	Before incubation	After 7 days of incubation				
		Control	[^{13}C]glucose		[^{13}C]glucose, H_2	
Taxonomy (phylum, class, family)	Light fraction	Light fraction	Light fraction	Heavy fraction	Light fraction	Heavy fraction
Actinobacteria, *Actinobacteria*,						
Acidimicrobiaceae	2	-	-	-	-	-
Aciditerrimonas-related phylotype[b]	2	2	3	-	1	-
Acidothermaceae	2	1	-	-	-	-
Demequinaceae	1	-	-	-	1	-
Microbacteriaceae	3	1	5	-	-	-
Micromonosporaceae	-	1	1	-	-	-
Nocardioidaceae	-	-	2	-	-	-
Solirubrobacteraceae	1	-	1	-	-	-
Firmicutes, *Bacilli*,						
Bacillaceae	-	-	3	-	1	-
Paenibacillaceae	-	-	3	2	2	-
Firmicutes, *Clostridia*,						
Anaerovorax-related phylotype[b]	-	2	-	-	-	1
Christensenellaceae	-	8	6	-	3	-
Clostridiaceae	-	4	1	-	4	-
Lachnospiraceae	-	27	29	57	29	49
Peptostreptococcaceae	47	3	4	-	6	-
Ruminococcaceae	-	33	18	24	33	17
Proteobacteria, *Alphaproteobacteria*,						
Bradyrhizobiaceae	1	-	-	-	-	-
Hyphomicrobiaceae	10	3	1	-	3	-
Methyloceanibacter-related phylotype[b]	2	-	-	-	-	-
Nordella-related phylotype[b]	-	-	2	-	-	-
Phyllobacteriaceae	4	-	1	-	1	-
Rhizobiaceae	2	-	3	-	1	-
Rhodobacteraceae	4	1	-	-	-	-
Acetobacteraceae	1	3	-	-	-	-
Rhodospirillaceae	1	-	2	-	-	-
Proteobacteria, *Gammaproteobacteria*,						
Enterobacteriaceae	2	2	10	17	7	32
Methylococcaceae	-	-	2	-	-	-

	Relative abundance of 16S rRNA sequences (%)[a]					
	Before incubation	After 7 days of incubation				
		Control	[^{13}C]glucose		[^{13}C]glucose, H_2	
Taxonomy (phylum, class, family)	Light fraction	Light fraction	Light fraction	Heavy fraction	Light fraction	Heavy fraction
Proteobacteria, *Deltaproteobacteria*,						
Desulfovibrionaceae	-	1	2	-	-	-
Labilithrix-related phylotype[b]	-	1	-	-	1	-
Phaselicystidaceae	-	1	2	-	-	-
Polyangiaceae	3	-	1	-	-	-
Planctobacteria, *Planctomycea*,						
Planctomycetaceae	8	3	2	-	5	1

[a] Assignment of 16S rRNA gene sequences to family-level phylotypes was based on an 87.5 % similarity cut-off (Yarza *et al.* 2008). RNA was obtained from gut contents before and after incubation of unsupplemented controls, [^{13}C]glucose-supplemented and [^{13}C]glucose-H_2-supplemented slurries. 16S rRNA sequences were obtained from light and heavy fractions of a cesium trifluoroacetate gradient. Values are rounded to the next whole number and thus may not sum up to 100 %. Legend: -, not detected.

[b] 16S rRNA sequences from gut contents shared at least 87.5 % similarity (i.e., family-level threshold) to the sequences of the given genus. Those genera are without hierarchical classification according to LPSN (www.bacterio.net) but cluster in phylogenetic trees within classes and phyla given in this table.

The relative abundance of *Ruminococcaceae* in heavy fractions was slightly higher than in light fractions derived from [^{13}C]glucose-supplemented slurries lacking supplemental H_2. Nevertheless, the relative abundance of *Ruminococcaceae* was (a) twice as high in light fractions than in heavy fractions derived from [^{13}C]glucose-supplemented slurries with supplemental H_2 but (b) higher in heavy fractions of [^{13}C]glucose-supplemented slurries lacking supplemental H_2 than in heavy fractions of [^{13}C]glucose-supplemented slurries with supplemental H_2, indicating that affiliated microorganisms might have assimilated ^{13}C in glucose-supplemented slurries lacking H_2.

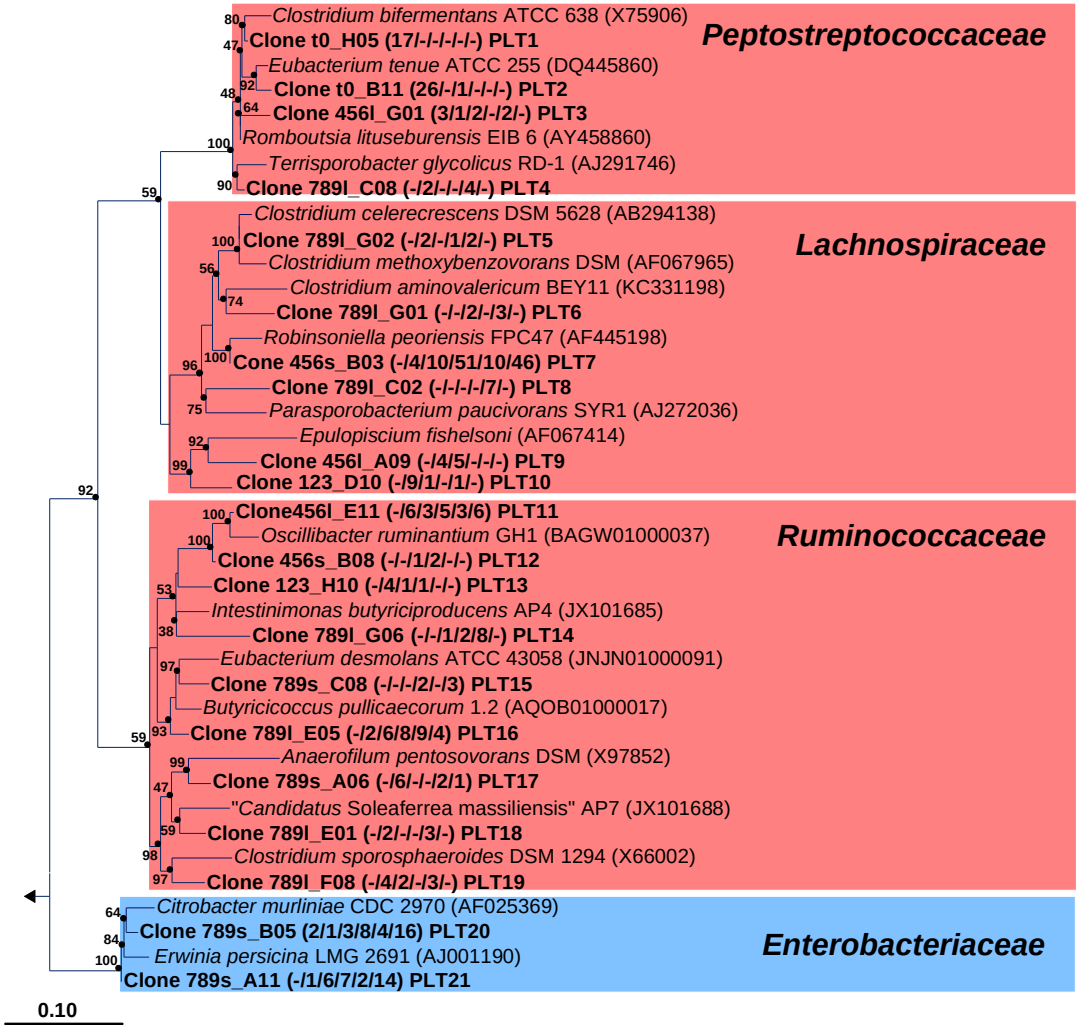


Figure 55: Phylogenic maximum parsimony tree of (a) representative 16S rRNA sequences of most abundant families retrieved from gut contents of *E. eugeniae* and (b) reference sequences.

Sequences derived from slurries with gut contents of *E. eugeniae* before and after seven days of anoxic incubation (Figure 51). The 16S rRNA gene sequences were based on a 97 % similarity cut-off clustered into family-level phylotypes (Yarza *et al.* 2008). Species-level phylotypes shown had a total relative abundance of at least 5 %. Accession numbers are indicated in brackets. Sequences correspond to nucleic acids 226-907 of the 16S rRNA gene of *E. coli* (AB035923). Filled dots indicate congruent nodes in the neighbor joining and the maximum likelihood tree. The 16S rRNA sequence of *M. kandleri* (M59932) was used as outgroup. The bar indicates a 0.1 change per nucleic acid. Bootstrap values are from the maximum parsimony tree (1,000 resamplings) and are only displayed at nodes congruent in all three trees. Relative abundances of species-level phylotypes in the clone libraries are given in parentheses in the following order: light fraction before incubation / light fraction after incubation of unsupplemented control / light fraction after incubation with [^{13}C]glucose / heavy fraction after incubation with [^{13}C]glucose / light fraction after incubation with [^{13}C]glucose and H_2 / heavy fraction after incubation with [^{13}C]glucose and H_2. Legend: PLT, phylotype; -, not detected. Values are rounded to nearest whole number.

5.5.6 Diversity of *fhs* phylotypes in [^{13}C]glucose-supplemented slurries

The detection of *fhs* from gradient-derived nucleic acids failed and thus, *fhs* sequences were obtained from DNA that was obtained from slurries after seven days of incubation (Figure 51). A total of 185 bacterial *fhs* sequences were obtained that clustered into 11 species-level phylotypes (Table 45). Rarefaction curves and coverage indicated that sampling was sufficient for species-level clustering of *fhs* phylotypes (Table 45, Figure 56). Diversity of species-level *fhs* phylotypes was mostly similar in unsupplemented controls and [^{13}C]glucose-supplemented slurries after the incubation (Table 45, Figure 56). The total detected diversity was higher than diversities in single slurries, indicating that some phylotypes were not unique to a specific treatment.

Table 45: Coverage of clone libraries, number of *fhs* sequences, and number of species-level *fhs* phylotypes obtained from anoxic slurries with gut contents of *E. eugeniae*.

Gene	Clone libraries[a]	No. of sequences	No. of phylotypes	Coverage
fhs	Control	60	6	98
	[^{13}C]glucose	62	9	98
	[^{13}C]glucose-H_2	63	7	97
	Total:	185	11	99

[a] *Fhs* sequences derived from anoxic slurries with gut contents of *E. eugeniae* after seven days of anoxic incubation (Figure 51). Nucleic acid extracts were analyzed before isopycnic centrifugation.

Detected *fhs* sequences were affiliated with *Clostridia*, *Bacilli*, and *Alphaproteobacteria* (Figure 57). Species-level *fhs* phylotype PLT4 was affiliated with *Clostridia* and was most abundant in unsupplemented slurries and showed a lower relative abundance in [^{13}C]glucose-supplemented slurries, indicating that affiliated microorganisms preferred experimental conditions in unsupplemented slurries (e.g., relatively low concentrations of organic acids, glucose, and H_2). Species-level *fhs* phylotypes PLT9 and PLT11 clustered most closely with *Mesorhizobium ciceri* (94 % and 77 % amino acid similarity, respectively, CP002447) and were only detected in [^{13}C]glucose-supplemented slurries lacking H_2, indicating that affiliated microorganisms were stimulated by supplemental glucose but seemed to be inhibited by high H_2 concentrations.

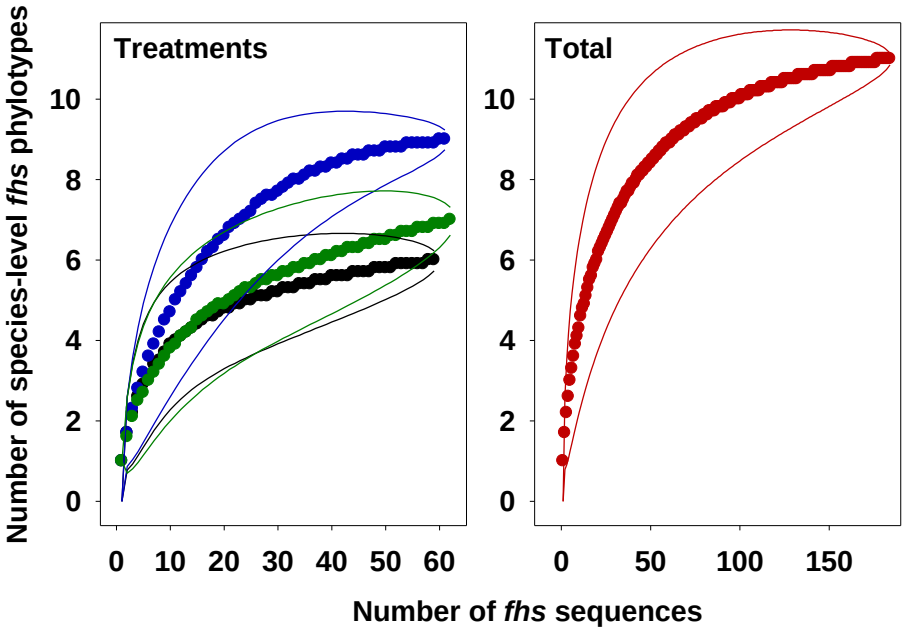


Figure 56: Rarefaction analysis of species-level *fhs* phylotypes obtained from gut contents of *E. eugeniae*.

Sequences derived from slurries with gut contents of *E. eugeniae* after seven days of anoxic incubation (Figure 51). *fhs* sequences were *in silico* translated into amino acids and based on a 76.4 % similarity cut-off clustered into species-level phylotypes (5.1.2). A 95 % confidence interval is shown. *fhs* sequences derived from anoxic slurries after incubation. Nucleic acid extracts were analyzed before isopycnic centrifugation. Color code: black, unsupplemented control; blue, [^{13}C]glucose-supplementation; green, [^{13}C]glucose-H_2-supplementation; red, total number of sequences.

Sequences of PLT9 and PLT11 overlapped with 156 amino acids and showed an amino acid sequences similarity of 86 %, indicating that PLT9 and PLT11 might actually derive from the same microorganism. Species-level *fhs* phylotype PLT10 was affiliated with *Clostridium celerecrescens* (98 % amino acid similarity, WP_038283002) and dominated in all treatments after incubation, especially [^{13}C]glucose-supplemented slurries with supplemental H_2. Species-level *fhs* phylotypes PLT2, PLT7, and PLT8 affiliated with the acetogen *Marvinbryantia formatexigens* (81 % amino acid similarity to PL2, WP_040782473), acetogen *Blautia hydrogenotrophica* (81 % amino acid similarity to PL2, WP_005953659), *Clostridium ultunense* (83 % amino acid similarity to PLT7, CP_005586003), and *Lysinibacillus manganicus* (90 % amino acid similarity to PLT8, WP_036183594), and were only observed in [^{13}C]glucose-supplemented slurries whereby slurries with supplemental H_2 showed a higher relative abundance than slurries lacking supplemental H_2, indicating that affiliated microorganisms were stimulated by supplemental glucose and supplemental H_2.

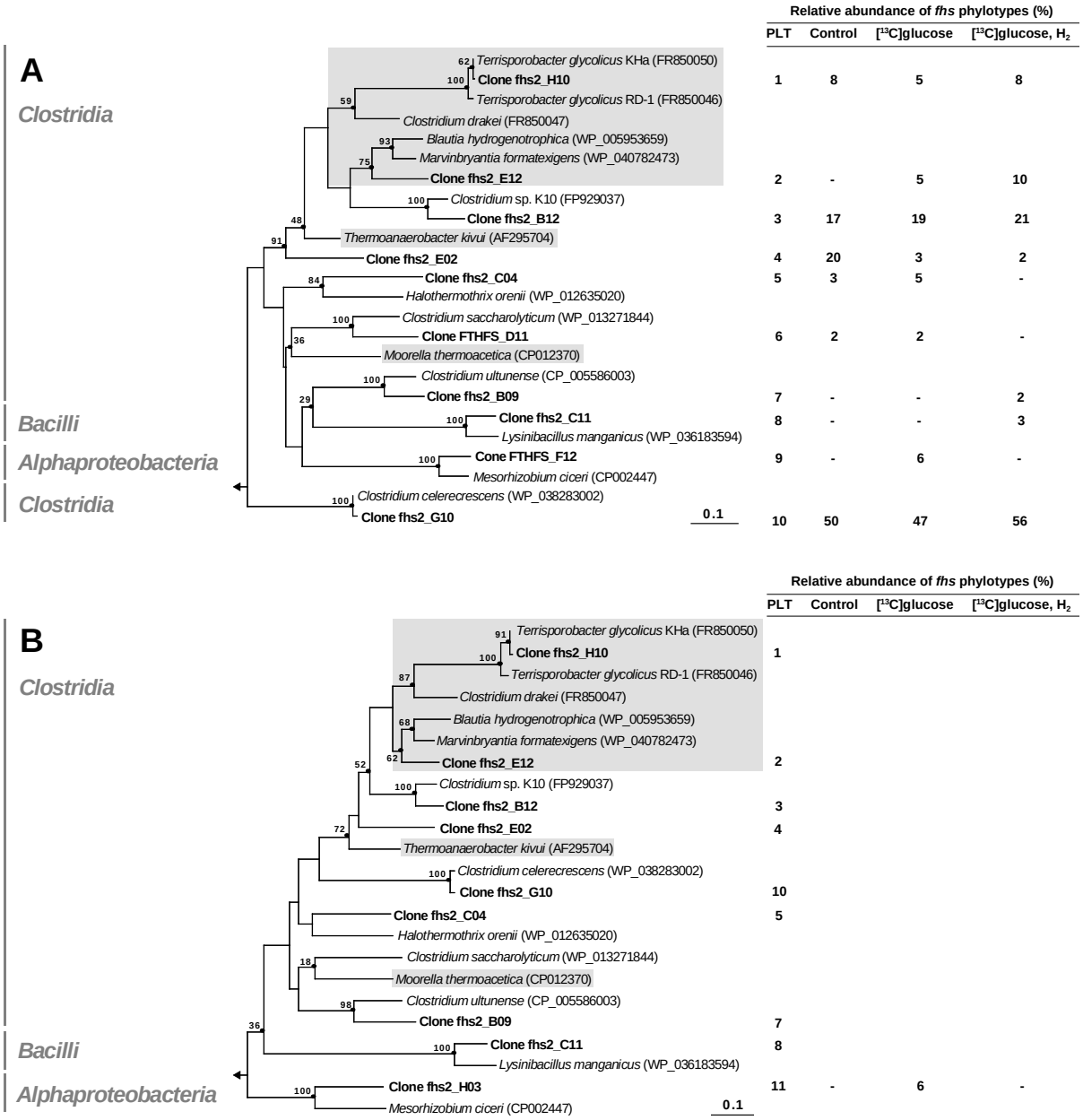


Figure 57: Phylogenic maximum parsimony trees of (a) representative *fhs*-encoded amino acid sequences retrieved from gut contents of *E. eugeniae* and (b) reference sequences.

Sequences were obtained from slurries with gut contents of *E. eugeniae* after seven days of anoxic incubation (Figure 51). *fhs* sequences were *in silico* translated into amino acids and based on a 76.4 % similarity cut-off clustered into species-level phylotypes (5.1.2). Accession numbers are indicated in brackets. Sequences in Panel A correspond to residues 150-377 and sequences in Panel B correspond to residues 237-422 of the *fhs*-encoded amino acid sequence of *M. thermoacetica* (CP012370). Filled dots indicate congruent nodes in the neighbor joining and the maximum likelihood trees. *M. labreanum* (CP000559) was used as outgroup. The bar indicates a 0.1 change per amino acid. Bootstrap values are from the maximum parsimony trees (1,000 resamplings) and are only displayed at nodes congruent in all three trees. Grey highlighting indicates sequences of acetogens. Legend: PLT, phylotype; -, not detected. Values are rounded to nearest whole number.

5.5.7 Properties of the acetogenic enrichment derived from gut contents

The methanogenic gut-derived enrichment converted H_2 (potentially together with CO_2) to acetate (5.5.2) and stimulation of acetate production due to supplemental H_2 in the RNA SIP analysis with supplemental [^{13}C]glucose (5.5.3, 5.5.6) were indicative for the presence of acetogens in gut contents of *E. eugeniae*. Acetogens were enriched from gut contents of *E. eugeniae* to analyze potential properties. An acetogenic enrichment EE was supplemented with H_2 or formate and incubated for 16 days under anoxic conditions. Acetogenic enrichment EE produced acetate as major end product together with traces of succinate, butyrate, lactate, propionate, formate, and ethanol in anoxic medium alone (4.4.5 without the supplementation of H_2 or formate). Supplemental H_2 and supplemental formate stimulated the production of acetate in a substrate to product ratio of 4:1 in each case (Figure 58), indicating for H_2- and formate-dependent acetogenesis.

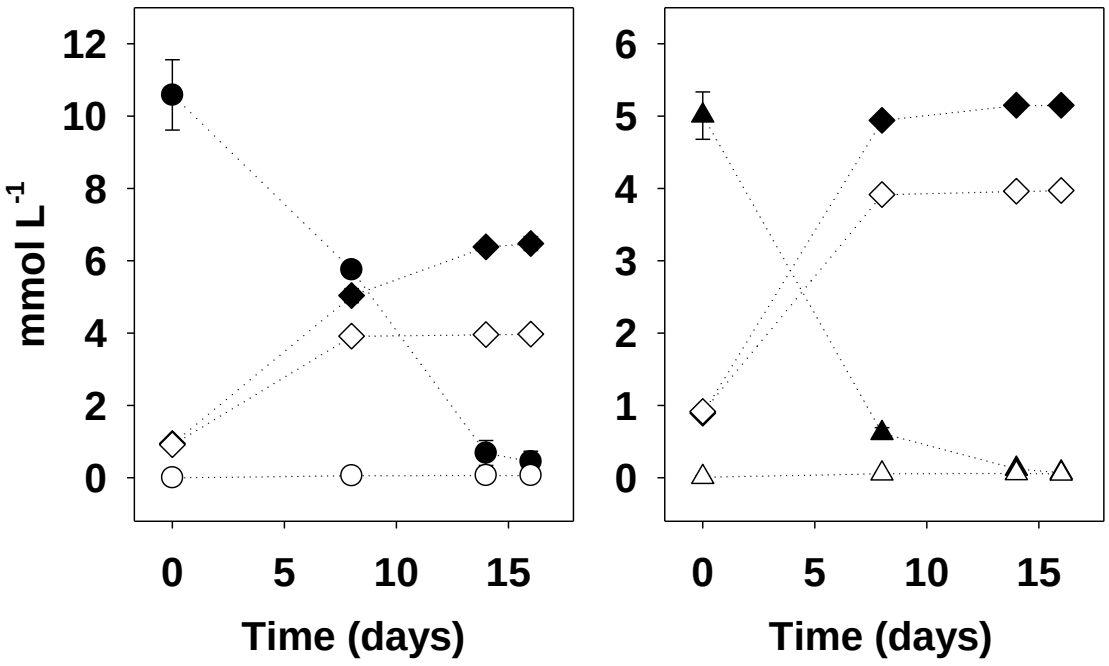


Figure 58: Effect of supplemental H_2 and formate on the product profiles of the acetogenic enrichment EE.

Incubation was performed at 25 °C. Legend: open symbols, unsupplemented control; closed symbols, H_2 or formate supplementation; circle, H_2; diamond, acetate; triangle, formate. Values are means of triplicate analysis. Error bars show the standard deviation.

The acetogenic enrichment stained Gram negative and consisted of uniform looking rods. Analysis of 16S rRNA genes indicated that the acetogenic enrichment consisted of two microorganisms, 80 % of sequences were closely related to the fermenters *Oscillibacter ruminantium* (99.6 % sequence similarity, NR_118156) and *Oscillibacter valericigenes* (96 % sequence similarity, NR_074793), and the other 20 % were closely related to the acetogens *Terrisporobacter glycolicum* strain mammoth-9 (100 % similarity, LN998075) and *Terrisporobacter mayombei* (99.2 % similarity, NR_104744). 16S rRNA gene sequences that were related to *O. ruminantium* were 99.6-99.9 % similar to each other. 16S rRNA gene sequences that were related to *Terrisporobacter glycolicus* were 99.4-99.9 % similar to each other.

5.6 Acetogenesis in aerated forest 'soil'

Although, acetogenesis is mostly known to occur in anoxic environments such as mire 'soils', rice field soils, and animal guts (Pester and Brune 2007, Liu and Conrad 2011, Hunger *et al.* 2015), aerated 'soils' also have the capacity to produce acetate (Küsel and Drake 1995, Peters and Conrad 1996) and acetogens have been isolated from such O_2-influenced habitats (Kuhner *et al.* 1997, Gößner *et al.* 1999). Hawaiian forest 'soil' from the Koke'e State Park produced acetate from H_2 (potentially with CO_2) under anoxic conditions, suggesting the presence of acetogens (Küsel *et al.* 2002).

An initial acetogenic culture KH consisted of very similar looking rods that converted numerous substrates (including xylan and raffinose) to mainly acetate under anoxic conditions and was thought be a pure culture. Xylan and raffinose are uncommon substrates for known acetogens (Drake *et al.* 2006), and it was suspected that KH was not a pure culture but consisted of at least two similar looking microorganisms. Serial dilutions of KH with undefined mineral medium UM4 (4.4.10) and supplementation with either H_2 or raffinose were prepared. The acetogen KHa and the fermenter KHb were obtained from the highest growth-positive dilution of H_2- and raffinose-supplemented tubes, respectively. A more detailed description of

utilized substrates, tolerance to salt, and sensitivity to O_2 than described below can be found in Hunger *et al.* (2011b).

5.6.1 Properties of the acetogenic isolate KHa obtained from forest 'soil'

Isolate KHa had a 99 % 16S rRNA gene similarity to *Terrisporobacter glycolicus* DSM1288 (X76750), a 97 % *fhs*-encoded amino acid sequence similarity to the acetogen *T. glycolicus* RD-1 (FR850046), and a 73 % *cooS*-encoded amino acid sequence similarity to *T. glycolicus* RD-1 (FR850055) (Figure 59). KHa grew anaerobically on H_2-CO_2, formate, ethanol, lactate, pyruvate, glucose, xylose, fructose, maltose, citrate, 1-propanol, n-butanol, and yeast extract, and formed predominantly acetate as end product together with traces of butyrate, ethanol, lactate, and H_2 (see Hunger *et al.* [2011b] for more details).

KHa consumed up to 1.5 % O_2 and tolerated up to 3 % O_2 in the gas phase and is capable of fermenting glucose under those conditions, illustrating the capacity of an acetogen to be O_2 tolerant. If exposed to low amounts of O_2, KHa produced acetate as main end product together with low amounts of ethanol, lactate, and H_2 from glucose. If grown under anoxic conditions, KHa produced acetate as main end product together with traces of butyrate and H_2 from glucose.

The substrate-product profile as well as the optimal growth conditions of KHa differed from those of fermentative *T. glycolicus* strains (Chamkha *et al.* 2001, Gaston and Stadtman 1963) but were similar to those of acetogenic *T. glycolicus* RD-1 (Küsel *et al.* 2001), indicating that KHa was a new strain of *T. glycolicus*.

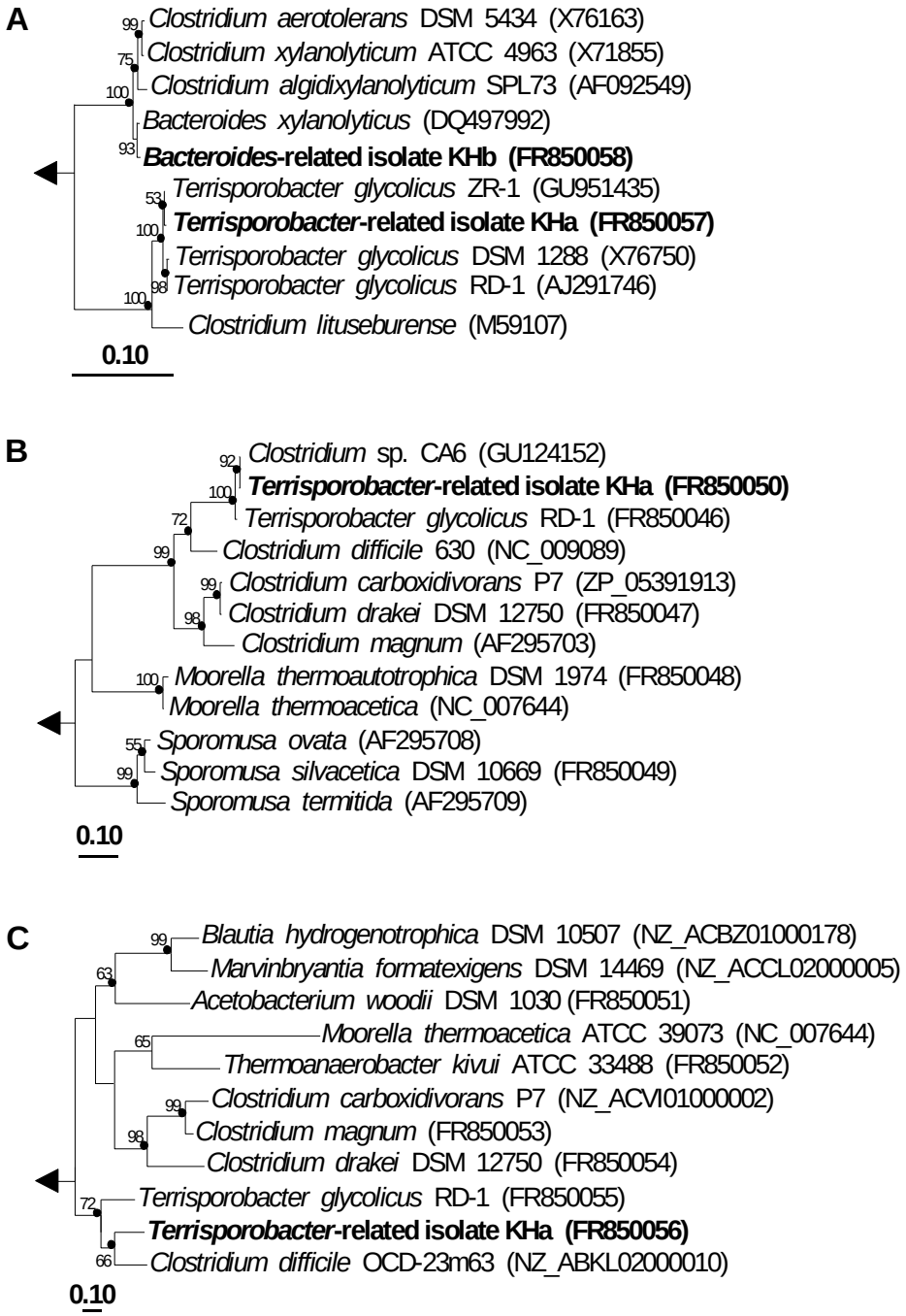


Figure 59: Phylogenic neighbor-joining trees of 16S rRNA gene sequences of KHa, KHb, and reference sequences (A), *in silico*-translated amino acid sequences encoded by *fhs* of KHa and reference sequences (B), and *in silico*-translated amino acid sequences encoded by *cooS* of KHa and reference sequences (C).

Accession numbers are indicated in brackets. Dots at nodes indicate the confirmation of tree topology by maximum likelihood and maximum parsimony calculations with the same data set. The bar indicates a 0.1 estimated change per nucleic acid or amino acid. The 16S rRNA gene sequence of *M. kandleri* (M59932), the *fhs*-encoded amino acid sequence of *M. labreanum* (CP000559), and the *cooS*-encoded amino acid sequence of *A. fulgidus* (NC_000917) were used as outgroups. Values next to the branches represent the percentages of replicate trees (>50 %) in which the associated taxa clustered together in the bootstrap test (1,000 bootstraps). Figure was modified from Hunger *et al.* (2011b).

5.6.2 Properties of the fermentative isolate KHb obtained from forest 'soil'

Isolate KHb had a 99 % 16S rRNA gene similarity to *Bacteroides xylanolyticus* (DQ497992) (Figure 59). A PCR signal with primers that target *fhs* or *cooS* was not obtained from isolate KHb. KHb grew anaerobically on xylan and raffinose and produced acetate, ethanol, lactate, formate, and H_2 as end products.

KHb consumed up to 4 % O_2 and tolerated up to 6 % O_2 in the gas phase during anoxic consumption of glucose and thus displayed a better tolerance to O_2 than KHa did. If exposed to low amounts of O_2, KHb produced (a) more acetate, lactate, and formate, and (b) less H_2 from glucose than under anoxic conditions.

The morphology and substrate-product profile of KHb were very similar to those of the type strain of *B. xylanolyticus* (Scholten-Koerselman *et al.* 1986), indicating that KHb was a new strain of *B. xylanolyticus*.

5.6.3 Effect of supplemental xylan on product profiles of isolates KHa and KHb

KHa and KHb derived from the enrichment KH that was capable of consuming xylan and producing acetate as the main end product under anoxic conditions. Isolate KHa cannot utilize xylan but KHb can. Isolate KHb fermented xylan to ethanol, H_2, and acetate under anoxic conditions (Figure 60).

A co-culture of KHb and KHa consumed xylan and released acetate as main end product with traces of ethanol and H_2 under anoxic conditions, suggesting a tight trophic link between acetogenic isolate KHa and fermentative isolate KHb.

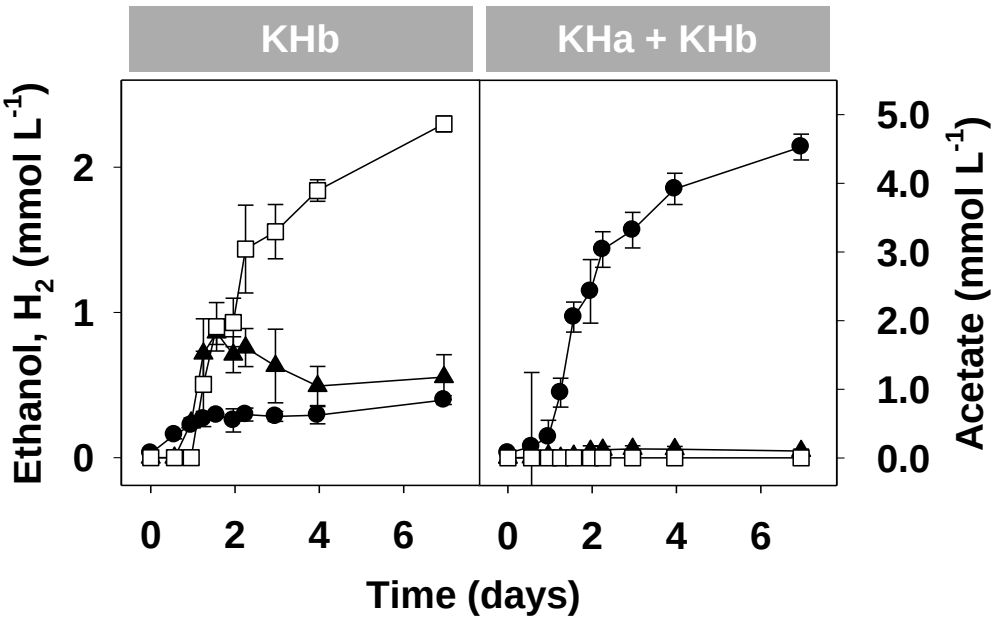


Figure 60: Effect of supplemental xylan on product profiles of KHb and co-cultures of KHa and KHb.

Incubation was performed at 30 °C. Values were corrected with values obtained from control cultures lacking xylan (i.e., *Bacteroides*-related isolate KHb alone and in co-culture with *Terrisporobacter*-related isolate KHa). Xylan was provided at a final concentration of approximately 0.1 % (w/v). The xylan stock solution was a sterile anoxic suspension prepared from autoclaved xylan powder. Symbols: filled circles, acetate; empty squares, ethanol; filled triangles, H_2. Values are means of triplicate analysis. Error propagations were plotted but were too little to appear as error bars. Figure was modified from Hunger *et al.* (2011b).

6 Discussion

Methanogenic food webs are widespread and can be found in natural and anthropogenic water-saturated habitats (Großkopf *et al.* 1998, Drake *et al.* 2009, Kato *et al.* 2015) such as mires, rice fields, or the alimentary canal of animals (Ohkuma *et al.* 1995, Yanagita *et al.* 2000). Those food webs have in common that a community of trophically linked anaerobes collectively produce CH_4 from organic polymers (Drake *et al.* 2009, Kato *et al.* 2015). The production of CH_4 by methanogenic archaea is a well-studied process in diverse CH_4-emitting habitats but the intermediary linked processes that precedes this production are less well understood and for most parts conceptualized rather than resolved (Zehnder 1978, McInerney and Bryant 1981, Drake *et al.* 2009). This dissertation contributes to the understanding of methanogenic food webs of mire 'soils', rhizospheres of mire-derived plants, and the alimentary canal of the earthworm *E. eugeniae*.

6.1 Fermentation and associated *Bacteria*

6.1.1 Contrasting mire 'soils'

All 'soils' of the contrasting mires fermented glucose (Figure 18). CO_2, butyrate, and acetate were the main fermentation products along with minor products such as H_2, ethanol, and propionate, a fermentation product profile similar to those observed with monosaccharide-supplemented Tundra wetland 'soil', monosaccharide-supplemented 'soil' from mire 2 in earlier studies, and rice straw-supplemented paddy 'soil' (Kotsyurbenko *et al.* 1996 [CO_2 not determined], Glissmann and Conrad 2000 [CO_2 not determined], Hamberger *et al.* 2008, Wüst *et al.* 2009a).

16S rRNA gene sequences affiliated with *Acidobacteriaceae*, *Clostridiaceae*, *Planctomycetaceae*, and *Veillonellaceae* increased in their relative abundance in 'soil' slurries due to the supplementation of glucose (Table 30). *Acidobacteriaceae* accounted for 25-41 % of the bacterial community in 'soils' of all acidic mires (Table 30). *Acidobacteriaceae*-affiliated

sequences were most closely related to *Telmatobacter*. *Telmatobacter* is adapted to moderately acidic pH, grows under microaerophilic and anoxic conditions, and ferments sugars (e.g., glucose, xylose) and polysaccharides (e.g., cellulose, cellobiose) to acetate, H_2, and traces of other compounds (Pankratov *et al.* 2012). *Acidobacteriaceae* have been labeled with [^{13}C]xylose in slurries with 'soil' of mire 2 in earlier studies (Hamberger *et al.* 2008). *Clostridiaceae* produce butyrate or acetate as major fermentation products together with other organic acids, alcohols, H_2, and CO_2, and can utilize a wide range of sugars and proteinaceous substrates (Wiegel 2009), and indeed were labeled with [^{13}C]glucose and [^{13}C]xylose in slurries with 'soil' from mire 2 in earlier studies (Hamberger *et al.* 2008). Many *Clostridiaceae*-affiliated sequences from mire 4 were related to the obligate anaerobes *Clostridium puniceum*, *Clostridium butyricum*, and *Clostridium gasigenes*. *C. puniceum*, *C. butyricum*, and *C. gasigenes* ferment sugars and produce acetate, butyrate, and H_2 as fermentation products (Lund *et al.* 1981, Wiegel 2009). Additional fermentation products are (a) lactate, formate, and butanol for *C. puniceum*, and (b) ethanol, lactate, and butanol for *C. gasigenes* (Lund *et al.* 1981, Wiegel 2009). Other 16S rRNA gene sequences within the *Clostridiaceae* were affiliated with the obligate anaerobes *Clostridium bowmanii*, *Clostridium frigidicarnis*, and *Clostridium hydrogeniformans*. *C. bowmanii*, *C. frigidicarnis*, and *C. hydrogeniformans* ferment carbohydrates such as glucose and produce butyrate, acetate, H_2 and CO_2 (Wiegel 2009, Bowman *et al.* 2010). Other fermentation products are (a) formate, ethanol, lactate, and butanol for *C. bowmanii*, and (b) ethanol, butanol, isobutyrate, isovalerate, oxaloacetate, and lactate for *C. frigidicarnis* (Wiegel 2009). *Planctomycetaceae*-affiliated sequences were related to *Schlesneria paludicola*. *S. paludicola* is a facultative aerobe that was isolated from peat bogs (Kulichevskaya *et al.* 2007). *S. paludicola* ferments carbohydrates such as glucose and maltose (Kulichevskaya *et al.* 2007). Members of *Veillonellaceae* ferment sugars predominantly to acetate, propionate, CO_2, and H_2 and smaller amounts of butyrate and succinate (Rainey 2009b).

Members of other families that were affiliated with detected 16S rRNA gene sequences are known to ferment sugars or other fermentable carbohydrates (i.e., *Anaerolineaceae*

[Yamada *et al.* 2006], *Bacillaceae* [Logan and De Vos 2009], *Chitinophagaceae* [Krieg *et al.* 2011], *Cytophagaceae* [Irgens 1977], *Holophagaceae* [Coates *et al.* 1999], *Ignavibacteriaceae* [Iino *et al.* 2010], *Methylocystaceae* [Xie and Yokota 2005, Madhaiyan *et al*. 2013], *Moraxellaceae* [Juni and Bøvre 2005], *Neisseriaceae* [Kwon *et al.* 2008], *Opitutaceae* [Chin *et al.* 2001], *Oxalobacteraceae* [Dehning and Schink 1989], *Rhodospirillaceae* [Sizova *et al.* 2007], *Ruminococcaceae* [Rainey 2009a], *Spirochaetaceae* [Paster 2011]) (Table 31). *Anaerolineaceae*-affiliated sequences were related to species of the obligate anaerobes *Leptolinea* and *Thermanaerothrix*. Members of *Leptolinea* hydrolyze polymers (e.g., starch) and ferment sugars such as glucose and xylose to predominantly lactate, acetate, pyruvate and H_2 with traces of succinate and formate (Yamada *et al.* 2006). Members of *Thermanaerothrix* hydrolyze polymers (e.g., xylan) and ferment sugars such as glucose and xylose to lactate, acetate, CO_2, and traces of H_2 (Grégoire *et al.* 2011). The *Bacillaceae*-affiliated sequence was related to species of *Bacillus*. Members of this genera can be facultative aerobes or obligate anaerobes that are capable of fermentation under anoxic conditions (Logan and De Vos 2009). Other members of the *Chitinophagaceae* are capable of fermentation and assimilation of sugars (Krieg *et al.* 2011). *Cytophagaceae*-affiliated sequences were related to *Meniscus glaucopis*. *M. glaucopis* is an aerotolerant anaerobe that ferments sugars such as raffinose, maltose, and glucose, and produces acetate, butyrate and succinate as end products (Irgens 1977). *Ignavibacteriaceae*-affiliated sequences were related to *Ignavibacterium album*. *I. album* is an obligate anaerobe that ferments sugars such as glucose and maltose (Iino *et al.* 2010). *Holophagaceae*-affiliated sequences were related with *Geothrix fermentans*. *G. fermentans* ferments citrate to predominantly acetate and succinate under the absence of alternative electron acceptors (Coates *et al.* 1999). Some *Methylocystaceae*-affiliated sequences were related to species of *Pleomorphomonas*. Species of *Pleomorphomonas* are facultative aerobes that are capable of glucose fermentation (Xie and Yokota 2005, Madhaiyan *et al.* 2013). Some *Moraxellaceae*-affiliated sequences were related to *Enhydrobacter aerosaccus*. *E. aerosaccus* is a facultative aerobe that ferments sugars such as glucose under anoxic conditions and grows best under microaerophilic

conditions (Juni *et al.* 2005). *Neisseriaceae*-affiliated sequences were related to *Paludibacterium*, a facultative aerobe that degrades cellulose and ferments glucose and other sugars (Kwon *et al.* 2008). *Opitutaceae*-affiliated sequences were related to *Opitutus terrae*. *O. terrae* is an obligate anaerobe that ferments mono-, di- and polysaccharides (e.g., glucose, cellobiose, starch) to predominantly acetate and propionate together with traces of succinate, lactate, formate, ethanol, and H_2 (Chin *et al.* 2001). *Oxalobacteraceae*-affiliated sequences were related to species of *Oxalobacter*. Members of *Oxalobacter* are anaerobes that ferment oxalate by decarboxylation and release formate and CO_2 (Dehning and Schink 1989, Garrity *et al.* 2005g). *Rhodospirillaceae*-affiliated sequences were related to *Telmatospirillum siberiense*. *T. siberiense* is a facultative aerobe that grows well under low pH conditions (Sizova *et al.* 2007). *T. siberiense* is capable of fermentation under anoxic condition and produces acetate, CO_2, and traces of formate from citrate (Sizova *et al.* 2007). *Ruminococcaceae* are obligate anaerobes that ferment various carbohydrates (e.g., glucose) to acetate, formate, butyrate, ethanol, H_2, and CO_2 (Rainey 2009a). *Spirochaetaceae*-affiliated sequences were related to species of *Spirochaeta*. *Spirochaetaceae* grow on carbohydrates or amino acids under anoxic or microaerophilic conditions and species of *Spirochaeta* ferment mostly di- and monosaccharides such as cellobiose and glucose, respectively (Paster 2011).

Secondary fermenters link primary fermentation with acetogenesis and methanogenesis via H_2 and formate (Jackson *et al.* 1999, Lengeler *et al.* 1999). 16S rRNA gene sequences related to secondary fermenters were detected in some mire ‘soils’ (i.e., *Syntrophaceae*, *Syntrophobacteraceae*, *Syntrophorhabdaceae*). *Syntrophaceae*-affiliated sequences were related to the obligate anaerobic species of *Syntrophus* and *Smithella*. Members of both genera are capable of fermenting crotonate, aromatic compounds or fatty acids such butyrate (Jackson *et al.* 1999, Kuever *et al.* 2005). *Syntrophobacteraceae*-affiliated sequences were related to species of *Syntrophobacter*. Species of *Syntrophobacter* are obligate anaerobes that ferment pyruvate, fumarate, malate, lactate or propanol (Kuever *et al.* 2005). *Syntrophorhabdaceae*-affiliated sequences were related to species of *Syntrophorhabdus*. Species of *Syntrophorhabdus* are anaerobes that degrade aromatic

compounds (Qiu *et al.* 2008). These taxa produce H_2, formate, and/or acetate as fermentation end products and grow best in a syntrophic relationship with a H_2-, formate-, and/or acetate-savaging microorganism such as methanogens and acetogens (does not use acetate) (Jackson *et al.* 1999, McInerney *et al.* 2008, Qiu *et al.* 2008), suggesting similar partnerships between detected secondary fermenters with acetogens and methanogens in analyzed mire 'soils'.

Based on the known properties of the detected taxa, fermentative taxa accounted for 26 %, 40 %, 38 %, and 63 % in the 'soils' of mires 1, 2, 3, and 4, respectively (Table 31), and might collectively drive the fermentation of glucose and other fermentable compounds in mire 'soils'.

6.1.2 The rhizosphere of mire plants

Plant roots release organic carbon and thereby generate an easily available carbon sources for microorganisms near the root zone (Walker *et al.* 2003). In this regard, unsupplemented soil-free roots released considerable more acetate, butyrate, propionate, and H_2 than unsupplemented root-free soil obtained from the same plant patches (5.4.1, 5.4.2, 5.4.3), indicating that (a) fermentation occurred in slurries with roots rather than with soils, and (b) fermentation of endogenous carbon might be a more pronounced process on roots than in soils potentially because of higher concentrations of endogenous plant-derived carbon at the root. 16S rRNA gene sequences affiliated with taxa that are known to ferment carbohydrates were detected on mire-derived roots (i.e., *Acidobacteriaceae* [Pankratov *et al.* 2012], *Bacteroidaceae* [Holdeman and Moore 1974], *Campylobacteraceae* [Luijten *et al.* 2003], *Chitinophagaceae* [Krieg *et al.* 2011], *Desulfuromonadaceae* [Schink 1984a], *Enterobacteriaceae* [Charrier *et al.* 2006], *Holophagaceae* [Coates *et al.* 1999], *Lachnospiraceae* [Parshina *et al.* 2003], *Marinilabiliaceae* [Zhao and Chen 2012], *Neisseriaceae* [Kwon *et al.* 2008], "*Nitrospiraceae*" [Henry *et al.* 1994], *Opitutaceae* [Chin *et al.* 2001], *Porphyromonadaceae* [Ueki *et al.* 2006], *Roseiarcaceae* [Kulichevskaya *et al.* 2014a], *Ruminococcaceae* [Rainey 2009a], *Veillonellaceae* [Ueki *et al.* 2014]) (Table 36,

Figure 43). Collectively, 68 % of detected taxa had the potential for fermentation, and might drive the fermentation of plant-derived carbohydrates in the rhizosphere of mire plants.

Holophagaceae and "*Nitrospiraceae*" were affiliated with the most abundant family-level 16S rRNA gene phylotypes before incubation of mire-derived roots and accounted each for 27 % of the bacterial community (Table 36). *Holophagaceae*-affiliated sequences were related to *Geothrix fermentans* (Figure 43). *G. fermentans* ferments citrate to acetate and succinate under the absence of alternative electron acceptors (Coates *et al.* 1999). "*Nitrospiraceae*"-affiliated sequences clustered with *Thermodesulfovibrio yellowstonii* (Figure 43). *T. yellowstonii* is an obligate anaerobe that ferments pyruvate (Henry *et al.* 1994). *Opitutaceae* and *Desulfuromonadaceae* were also detected on roots before incubation (Table 36). One 16S rRNA gene sequence was most closely related to *Opitutus terrae* (*Opitutaceae*), an anaerobe that was isolated from paddy soil (Chin *et al.* 2001). *O. terrae* ferments sugars to predominantly propionate and acetate along with succinate, lactate, formate, ethanol and H_2 (Chin *et al.* 2001). *Desulfuromonadaceae*-affiliated sequences were related to *Pelobacter propionicus* (Table 36, Figure 43). *P. propionicus* is an obligate anaerobe that ferments C_2 compounds such as 2,3-butanediol to acetate and propionate (Schink 1984a). *P. propionicus* utilizes also other alcohols, lactate and pyruvate (Schink 1984a).

Bacteroidaceae, *Enterobacteriaceae*, and *Veillonellaceae* were affiliated with the most abundant phylotypes that were detected after the anoxic incubation of unsupplemented and of formate-H_2-supplemented roots (Table 36). *Bacteroidaceae*-affiliated sequences were related to *Bacteroides eggerthii* (Figure 43). *B. eggerthii* is an anaerobe that ferments sugars to succinate and acetate (Holdeman and Moore 1974). *Enterobacteriaceae*-affiliated sequences were related to *Buttiauxella gaviniae* (Figure 43). *B. gaviniae* oxidizes lactose and acetate under oxic conditions (Müller *et al.* 1996) whereby other species of *Buttiauxella* ferment cellobiose, maltose, and hydrolyze chitin (Charrier *et al.* 2006). *Veillonellaceae*-affiliated sequences were related to *Propionispira paucivorans* and *Propionispira raffinosivorans* (Figure 43). *P. paucivorans* and *P. raffinosivorans* are obligate anaerobes that ferment sugars such

as glucose and organic acids such as lactate or succinate to acetate and propionate (Ueki *et al.* 2014). *Neisseriaceae*-affiliated sequences were also detected after anoxic incubation of unsupplemented and formate-H_2-supplemented roots and were related to *Paludibacterium yongneupense* (Table 36, Figure 43). *P. yongneupense* is a facultative aerobe that was isolated from wetland peat and can grow at pH 4.0 (Kwon *et al.* 2008). *P. yongneupense* hydrolyzes starch and cellulose, and ferments glucose (Kwon *et al.* 2008).

Lachnospiraceae-affiliated sequences were detected before and after anoxic incubation of unsupplemented and formate-H_2-supplemented roots and were related to *Clostridium amygdalinum* (Table 36, Figure 43). *C. amygdalinum* is aerotolerant and ferments a wide range of carbohydrates such as glucose (Parshina *et al.* 2003). Fermentation of glucose yields acetate, ethanol, H_2 and CO_2 (Parshina *et al.* 2003).

Campylobacteraceae and *Chitinophagaceae* were detected after anoxic incubation of unsupplemented mire-derived roots (Table 36). The *Campylobacteraceae*-affiliated sequence was related to *Sulfurospirillum halorespirans* (Figure 43). *S. halorespirans* ferments fumarate and pyruvate (Luijten *et al.* 2003). One *Chitinophagaceae*-affiliated sequence was related to *Chitinophaga terrae* (Figure 43). *C. terrae* is an aerobe that was isolated from soil and grows on sugars (Kim and Jung 2007). Other members of the *Chitinophagaceae* are capable of fermentation and assimilation of sugars (Krieg *et al.* 2011).

Acidobacteriaceae-affiliated sequences were detected before and after anoxic incubation of formate-H_2-supplemented roots and were related to "*Candidatus* Koribacter versatilis" (Table 36, Figure 43). "*Candidatus* K. versatilis" has the potential to degrade polymers (e.g., cellulose), sugars (e.g., glucose), amino acids, and alcohols based on genome studies (Elkins *et al.* 2008). Other members of *Acidobacteriaceae* such as *Telmatobacter* ferment sugars to predominantly acetate and H_2 (Pankratov *et al.* 2012).

Marinilabiliaceae, *Porphyromonadaceae*, *Roseiarcaceae*, and *Ruminococcaceae*, were detected after anoxic incubation of formate-H_2-supplemented roots (Table 36). One *Marinilabiliaceae*-affiliated sequences was related to *Alkalitalea saponilacus* (Figure 43). *A.*

saponilacus is an anaerobe that ferments sugars and polymers to predominantly acetate and propionate (Zhao and Chen 2012). *Porphyromonadaceae*-affiliated sequences were related to *Paludibacter propionicigenes* (Figure 43). *P. propionicigenes* was isolated from rice plant residues in an anoxic rice field soil (Ueki *et al.* 2006). *P. propionicigenes* ferments various di- and monosaccharides such as cellobiose or glucose, respectively, to acetate and propionate (Ueki *et al.* 2006). *Roseiarcaceae*-affiliated sequences were related to *Roseiarcus fermentans* (Figure 43). *R. fermentans* was isolated from *Sphagnum* peat and preferably ferments sugars (e.g., glucose, fructose) and organic acids (e.g., succinate, malate) under microaerophilic conditions and a pH of 5.5-6.5 (Kulichevskaya *et al.* 2014a). Propionate, acetate, and H_2 are released as major fermentation end products from fructose (Kulichevskaya *et al.* 2014a). *Ruminococcaceae*-affiliated sequences were related to *Clostridium sporosphaeroides* (Figure 43). *C. sporosphaeroides* produces predominantly acetate, butyrate, and H_2 together with small amounts of propionate as fermentation end products from amino acids (Rainey 2009a, Cibis *et al.* 2016). Growth on glucose is weak and propionate is produced from lactate (Rainey 2009a, Cibis *et al.* 2016).

Members of the *Enterobacteriaceae*, *Lachnospiraceae*, and *Carnobacteriaceae* were not detected with ‘soil’ or soil slurries of contrasting mires but were isolated from a mixture of mire-derived roots. *Carnobacteriaceae* were not detected in ‘soils’ of contrasting mires or with mire-derived roots (5.2.8, 5.4.6). *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 (*Enterobacteriaceae*) fermented glucose, arabinose, and citrate to ethanol, organic acids, CO_2, and H_2 (5.4.7, 5.4.8). Root-derived *Clostridium*-related isolate SB3 (*Lachnospiraceae*) and *Carnobacterium*-related isolate SB4 (*Carnobacteriaceae*) fermented glucose to ethanol, acetate, formate, and lactate. *Clostridium*-related isolate SB3 additionally produced H_2.

Isolate SB1 was related to *Citrobacter braakii* and *Citrobacter freundii* (Figure 45). Members of *Citrobacter* are facultative aerobes that grow via respiration or fermentation and stain Gram negative (Brenner *et al.* 2005). *C. freundii* and *C. braakii* utilize arabinose, glucose,

and citrate, and produce acid and gas from glucose fermentation (Brenner *et al.* 2005). Described properties of *C. freundii* and *C. braakii* have been also observed with *Citrobacter*-related isolate SB1 (5.4.7,), indicating that isolate SB1 is a species of *Citrobacter*. All species of *Citrobacter* react Catalase positive (Brenner *et al.* 2005) but *Citrobacter*-related isolate SB1 reacted repeatedly catalase negative. Further investigations will have to determine if the tests for catalase activity failed or if isolate SB1 belongs to another genus.

Isolate SB2 was related to *Hafnia alvei* and *Hafnia paralvei* (Figure 45). *H. paralvei* and *H.* alvei stain Gram negative, react oxidase negative and catalase positive, and form motile rods (Brenner *et al.* 2005, Janda and Abbott 2006, Huys *et al.* 2010). Both species produce acid from glucose and arabinose (Brenner *et al.* 2005, Janda and Abbott 2006, Huys *et al.* 2010). Some strains of *H.* alvei utilize citrate (Janda and Abbott 2006). Described properties of *H. paralvei* and *H.* alvei have been also observed with *Hafnia*-related isolate SB2 (5.4.7), indicating that isolate SB2 is a species of *Hafnia*.

Isolate SB3 was related to *Clostridium celerecrescens* and *Clostridium sphenoides* (Figure 46). *C. celerecrescens* and *C. sphenoides* are obligate anaerobes (Palop *et al.* 1989, Rainey 2009a). Isolate SB3 did not grow under oxic conditions, indicating that isolate SB3 is an anaerobe. It cannot be ruled out that growth under oxic conditions was inhibited by the low pH (i.e., pH 5.0) or that isolate SB3 tolerates low concentrations of O_2 but not high concentrations. *C. celerecrescens* reacts catalase negative (Palop *et al.* 1989), as *Clostridium*-related isolate SB3 did. *C. celerecrescens* stains Gram positive, is motile, utilizes glucose, and produces ethanol, acetate, formate, butyrate, lactate, succinate, CO_2, and H_2 as major fermentation products from cellulose or cellobiose (Palop *et al.* 1989, Rainey 2009a). Cellulose and cellobiose consist of glucose molecules (Kokorevics *et al.* 1997, Yang *et al.* 2015) and should stimulate similar product profiles than glucose. *C. sphenoides* stains Gram negative, occurs single, paired or on chains, and is motile (Rainey 2009a), as *Clostridium*-related isolate SB3 did. *C. sphenoides* ferments carbohydrates such as cellobiose, maltose, and citrate to predominantly acetate, formate, CO_2, and H_2 and small amounts of lactate,

succinate, and ethanol (Rainey 2009a). Fermentation profile of *Clostridium*-related isolate SB3 from glucose was similar (5.4.8) to the fermentation profiles of *C. celerecrescens* and *C. sphenoides* with the difference that *Clostridium*-related isolate SB3 did not produce butyrate or succinate as *C. celerecrescens* and *C. sphenoides* (also no production of butyrate) did (Palop *et al.* 1989), indicating that *Clostridium*-related isolate SB3 might be a new species.

Next cultured relative of isolate SB4 was *Carnobacterium maltaromaticum* (Figure 46). Isolate SB4 grew minimal under oxic conditions and did not consume supplemental formate, citrate, or glucose, indicating that isolate SB4 is a facultative aerobe, as *C. maltaromaticum* is (Mora *et al.* 2003). Growth under oxic conditions was likely inhibited by the low pH (i.e., pH 5.0). *C. maltaromaticum* stains Gram positive and forms rods that can appear single or in chains (Mora *et al.* 2003). Oxidase and catalase reactions are negative (Mora *et al.* 2003). Acetate, lactate, and ethanol are fermentation end products from glucose (Mora *et al.* 2003). Gas production is weak and might in some cases be non-detectable (Mora *et al.* 2003). Above mentioned properties of *C. maltaromaticum* are shared with *Carnobacterium*-related isolate SB4. *Carnobacterium*-related isolate SB4 is motile in contrast to *C. maltaromaticum* that is non-motile. Motility is not uncommon to *Carnobacterium* species. For example, *Carnobacterium mobile* is motile (Collins *et al.* 1987). Those properties indicate that SB4 is similar but not identical to *C. maltaromaticum* and may be a new species.

6.1.3 The gut of the earthworm *E. eugeniae*

The mucus of the earthworm gut is rich on polysaccharides and hydrolyzed mucus consists of diverse sugars (Rahemtulla and Løvtrup 1975, Wüst *et al.* 2009b). For example, approximately 10 mM glucose and other saccharides such as arabinose, fucose, galactose, isomaltose, maltose, mannose, or rhamnose can be detected in hydrolyzed mucus (Wüst *et al.* 2009b). Glucose serves as a potential carbon source for earthworm gut-derived microorganisms (Wüst *et al.* 2011, Schulz *et al.* 2015). Slurries with gut contents of the earthworm *E. eugeniae* rapidly consumed supplemental glucose and produced acetate, succinate, propionate, butyrate, formate, CO_2, and H_2 (5.5.3), indicating the presence of active

fermenters in the earthworm gut. A similar glucose-driven fermentation profile was observed with gut contents of *L. terrestris* and gut contents of *E. eugeniae* in earlier studies (Wüst *et al.* 2011, Schulz *et al.* 2015). *Enterobacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* were the most abundant phylotypes detected in gut contents of *E. eugeniae* (Table 44). Several species within those and other family-level 16S rRNA phylotypes are known to be capable of fermentation (e.g., species within *Anaerovorax*-related phylotype, *Christensenellaceae*, *Clostridiaceae*). Those taxa were capable of producing the above mentioned organic acids and gases from glucose. Taken together, taxa that are capable of fermenting glucose or other sugars had a relative abundance of approximately 49 % in the light fraction before treatment (Table 44) and might collectively drive the fermentation of mucus- and substrate-derived sugars in the earthworm gut.

Lachnospiraceae-affiliated sequences were detected in all slurries after the anoxic incubation of gut contents and were labeled by [^{13}C]glucose-derived carbon (Table 44, Figure 55). Members of *Lachnospiraceae* were also identified as glucose fermenters in an earlier study with gut contents of *E. eugeniae* (Schulz *et al.* 2015), reinforcing the likelihood of this taxa to be involved in fermentation of sugars in the gut of *E. eugeniae*. Most *Lachnospiraceae*-affiliated 16S rRNA sequences were related to sequences of (a) obligate anaerobes that are capable of fermentation (e.g., *Clostridium celerecrescens*, *Clostridium aminovalericum*, *Robinsoniella peoriensis*), (b) the anaerobe *Parasporobacterium paucivorans*, and (c) the symbiont *Epulopiscium fishelsoni* (Figure 55). Sequences affiliated with *R. peoriensis* accounted for about 50 % of total detected taxa in heavy fractions of [^{13}C]glucose-supplemented slurries (Figure 55) and were labeled by [^{13}C]glucose-derived carbon. *R. peoriensis* was isolated from swine manure (Cotta *et al.* 2009). *R. peoriensis* hydrolyzes polymers (e.g., starch) and ferments sugars such as glucose or arabinose to predominantly acetate and succinate together with traces of formate and lactate (Cotta *et al.* 2009). *C. aminovalericum* ferments various carbohydrates including amino acids and sugars such as glucose or arabinose (Hardman and Stadtman 1960). Not only 16S rRNA sequences but also about half of the detected *fhs* sequences were affiliated with *C. celerecrescens* (PLT10, Figure

57). *C. celerecrescens* was isolated from cow manure (Palop *et al.* 1989). Cow manure was also the substrate on which *E. eugeniae* was raised on before the gut contents was obtained for analysis (4.3). *C. celerecrescens* ferments sugars such as glucose, arabinose, or mannose (Palop *et al.* 1989). Fermentation products are ethanol, acetate, formate, butyrate, lactate, succinate, CO_2, and H_2 (Palop *et al.* 1989). *P. paucivorans* grows on methoxylated aromatic compounds and sulfide, and produces acetate and butyrate together with dimethyl sulfide and methanethiol (Lomans *et al.* 2001). *P. paucivorans* is not capable of utilizing common carbohydrates such as glucose (Lomans *et al.* 2001). *E. fishelsoni* was isolated from the gut of a reef fish and is a symbiont of this fish (Montgomery and Pollak 1988). Species-level phylotypes that were related to the glucose fermenting taxa *C. aminovalericum* and *C. celerecrescens* were most often detected in light or heavy fraction after supplementation of gut contents with [^{13}C]glucose, and species-level phylotype PLT7 related to the glucose fermenting taxa *R. peoriensis* was labeled by [^{13}C]glucose-derived carbon (Figure 55), indicating the capacity of those phylotypes to ferment glucose in gut contents of *E. eugeniae*.

Enterobacteriaceae-affiliated sequences were labeled by [^{13}C]glucose-derived carbon in slurries with supplemental [^{13}C]glucose-H_2 and in slurries with supplemental [^{13}C]glucose without H_2 (Table 44, Figure 55), and were related to the facultative aerobes *Citrobacter murliniae* and *Erwinia persicina*. *Enterobacteriaceae* were also identified as glucose fermenters in gut contents of the earthworm *L. terrestris* (Wüst *et al.* 2009b). *C. murliniae* uses sugars (e.g., glucose), amino acids, and organic acids (e.g., propionate) for growth (Brenner *et al.* 1999). *E. persicina* utilizes sugars such as glucose and arabinose, a few amino acids, and a few organic acids such as acetate (Hao *et al.* 1990). Fermentation of glucose yields succinate, lactate, formate and acetate (Brenner *et al.* 2005). The metabolic potentials of *C. murliniae* and *E. persicina*, indicated that the related taxa fermented glucose in slurries with gut contents of *E. eugeniae* and might have been additionally stimulated by high concentrations of organic acids such as acetate and propionate.

Ruminococcaceae-affiliated sequences were detected after the anoxic incubation with supplemental [^{13}C]glucose and might be minorly labeled with [^{13}C]glucose-derived carbon (Table 44). *Ruminococcaceae* were also minorly labeled by [^{13}C]glucose-derived carbon in another study with gut contents of *E. eugeniae* (Schulz *et al.* 2015). Detected phylotypes were related to sequences of the obligate anaerobes *Oscillibacter ruminantium*, *Intestinimonas butyriciproducens*, *Eubacterium desmolans*, *Butyricicoccus pullicaecorum*, *Anaerofilum pentosovorans*, and *Clostridium sporosphaeroides*, and "*Candidatus* Soleaferrea massiliensis" (Table 44, Figure 55). *O. ruminantium*, *I. butyriciproducens*, *B. pullicaecorum*, *A. pentosovorans*, and *C. sporosphaeroides* ferment sugars such as glucose (Zellner *et al.* 1996, Eeckhaut *et al.* 2008, Rainey 2009a, Kläring *et al.* 2013, Lee *et al.* 2013b, Cibis *et al.* 2016). *O. ruminantium* produces predominantly butyrate together with traces of acetate, ethanol and butanol as fermentation end products (Lee *et al.* 2013b). *I. butyriciproducens* produces predominantly butyrate and acetate together with traces of lactate, isovalerate, and valerate as fermentation end products (Kläring *et al.* 2013). *B. pullicaecorum* produces predominantly butyrate together with H_2 and CO_2 as fermentation end products (Eeckhaut *et al.* 2008). *A. pentosovorans* produces lactate, acetate, ethanol, formate, and CO_2 as fermentation end products (Zellner *et al.* 1996). *C. sporosphaeroides* ferments amino acids well and glucose weakly, and produces predominantly acetate, butyrate, and H_2 together with small amounts of propionate (Rainey 2009a, Cibis *et al.* 2016). *E. desmolans* was isolated from cat feces (Morris *et al.* 1986). *E. desmolans* ferments inositol and produces predominantly acetate and butyrate with traces of succinate and lactate but cannot ferment common sugars (Morris *et al.* 1986). "*Candidatus* Soleaferrea massiliensis" is an anaerobe with hitherto unknown metabolic potentials (Pfleiderer *et al.* 2013). Most detected species-level phylotypes that were affiliated with *Ruminococcaceae* are capable of fermenting glucose but only phylotypes related to *O. ruminantium* and *B. pullicaecorum* were slightly more abundant in heavy fractions than in light fractions and could potentially be labeled by [^{13}C]glucose.

Peptostreptococcaceae-affiliated sequences had a relative abundance of 47 % in light fractions before the anoxic incubation but were also detected in light fractions after incubation

with supplemental [^{13}C]glucose (Table 44). Detected phylotypes were related to obligate anaerobic taxa that are capable of fermentation: *Clostridium bifermentans*, *Eubacterium tenue*, and *Romboutsia lituseburensis* (Figure 55). *C. bifermentans* and *E. tenue* ferment sugars such as glucose weakly (Ludwig *et al.* 2009, Wiegel 2009), a possible explanation why related phylotypes were mostly detected before incubation with [^{13}C]glucose (Figure 55). *C. bifermentans* produces predominantly acetate, formate, and H_2 together with traces of butyrate and propionate as fermentation end products (Wiegel 2009). *E. tenue* produces acetate, formate, and H_2 together with traces of propionate, ethanol and other alcohols as fermentation end products (Ludwig *et al.* 2009). *R. lituseburensis* ferments sugars including glucose to predominantly formate and acetate together with traces of lactate and propionate (Holdeman *et al.* 1977, Gerritsen *et al.* 2014). Detected phylotypes that were affiliated with *Peptostreptococcaceae* are potentially capable of fermenting sugars but seemed to play a minor role in glucose fermentation in anoxic slurries with gut contents of *E. eugeniae*.

Sequences affiliated with *Christensenella minuta* (*Christensenellaceae*) were detected in light fractions of the unsupplemented control and of [^{13}C]glucose-supplemented slurries after the incubation (Table 44). *C. minuta* ferments various sugars such as arabinose, xylose, and glucose to predominantly acetate and butyrate (Morotomi *et al.* 2012), indicating that detected phylotypes were potentially capable of glucose fermentation but seemed to play a minor role in the fermentation of glucose in anoxic slurries with gut contents of *E. eugeniae* and might have grown on other sugars.

Clostridiaceae-affiliated sequences were detected in light fractions of the unsupplemented control and in light fractions of [^{13}C]glucose-supplemented slurries after the incubation (Table 44, Figure 55), and were related to *Clostridium sartagoforme*, *Clostridium disporicum*, *Clostridium celatum*, and *Clostridium puniceum*. In contrast, *Clostridiaceae* were labeled with [^{13}C]glucose in gut contents of the earthworm *L. terrestris* (Wüst *et al.* 2009b). *C. sartagoforme*, *C. disporicum*, *C. celatum*, and *C. puniceum* are obligate anaerobes that ferment various carbohydrates such as glucose or cellobiose to gas and acid (Partansky and

Henry 1935, Hauschild and Holdeman 1974, Lund *et al.* 1981, Horn 1987). For example, *C. disporicum* produces predominantly acetate and lactate with traces of succinate, butyrate, and ethanol (Horn 1987), and *C. celatum* produces predominantly acetate, formate, ethanol, and H_2 with traces of butyrate (Hauschild and Holdeman 1974). Related phylotypes that were detected in gut contents of *E. eugeniae* were potentially capable of glucose fermentation but were not labeled under the experimental conditions, indicating that detected taxa played a minor role in the fermentation of glucose in anoxic incubations, and since phylotypes were not detected before the incubation they might also play a minor role in gut contents of the earthworm *E. eugeniae*.

A few sequences affiliated with *Anaerovorax odorimutans* were detected in light and heavy fractions before and after supplementation with [^{13}C]glucose. *A. odorimutans* is an obligate anaerobe and ferments only butane-1,4-diamine, 4-aminobutyrate, and 4-hydroxybutyrateamino acids and produced butyrate, acetate and H_2 (Matthies *et al.* 2000), indicating that related phylotypes that were detected in gut contents of *E. eugeniae* were unlikely involved in the fermentation of glucose.

6.2 Formation of H_2 from formate and associated taxa

Formate can be reversibly transformed to H_2 and CO_2 ($HCOO^- + H^+ \leftrightarrow H_2 + CO_2$) by enzymes such as the FHL complex that consists of a formate dehydrogenase and a hydrogenase (Trchounian and Sawers 2014). This reaction has a Gibbs free energy under standard conditions close to that of thermodynamic equilibrium ($\Delta G^{0\prime} \approx 1$ kJ mol^{-1}). However, the formation or consumption of formate can be thermodynamically favorable under physiological conditions and subsequently be coupled to energy conservation (Andrews *et al.* 1997, Dolfing *et al.* 2008, Kim *et al.* 2010, Lim *et al.* 2012). For example, *Thermococcus onnurineus* and other species of *Thermococcus* are capable to oxidize formate with H^+ as electron acceptor, form H_2 and CO_2, and conserve enough energy for growth under hyperthermophilic conditions (Kim *et al.* 2010). The formation of H_2 from supplemental formate has been observed with mire ‘soil’ (Figure 28), soil-free roots of different plant species, and

root-free soils from the same patches were roots derived from (Figure 33, Figure 34, Figure 35).

The first two formate pulses stimulated predominantly the production of H_2 and subsequent pulses stimulated additionally the production of acetate and CH_4 in slurries with mire 'soil' (Figure 28), indicating that H_2 was formed from formate and quickly utilized by H_2-scavenging microorganisms. *Rhodospirillum*-affiliated microorganisms (*Rhodospirillaceae*) were identified in those slurries as potential FHL-containing taxa that formed formate-derived H_2 (Hunger *et al.* 2011a). 16S rRNA sequences affiliated with *Rhodocyclaceae* were detected in formate-supplemented 'soil' slurries (Hunger *et al.* 2011a). Some members of this family such as *Rhodocyclus*-affiliated species are capable of photoheterotrophic growth with H_2 as electron donor under anoxic conditions (Garrity *et al.* 2005h), indicating that affiliated microorganisms might have utilized some of the H_2 before acetogenesis and methanogenesis was active at times of incubation when slurries were exposed to light (e.g., during gas analysis).

The formation of formate-derived H_2 was a major process in slurries with *Carex* and *Molinia* roots from mire 2 (5.4.1). Nearly equimolar amounts of H_2 were formed by *Carex* and *Molinia* roots in response to an initial pulse of formate (Figure 33, Figure 34, Figure 35). *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 (*Enterobacteriaceae*) derived from a mixture of *Carex* and *Molinia* roots and were capable to form H_2 from formate (Figure 44). The saccharolytic fermenter *Escherichia coli* (*Enterobacteriaceae*) oxidizes formate to CO_2 with a formate dehydrogenase ($HCOO^- \rightarrow CO_2 + H^+ + 2e^-$) and subsequently reduces the protons to H_2 with a membrane-bound hydrogenase ($2H^+ + 2e^- \rightarrow H_2$) (Böhm *et al.* 1990, Andrews *et al.* 1997), suggesting that the formation of H_2 from formate occurred in a similar manner in *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2. *E. coli* has two FHL complexes that consist of two different group 4 [NiFe]-hydrogenases, hydrogenase 3 (Hyc) and hydrogenase 4 (Hyf) (Peck and Gest 1957, Böhm *et al.* 1990, Sauter *et al.* 1992, Andrews *et al.* 1997, Bagramyan *et al.* 2002). Genes that encode group 4 [NiFe]-hydrogenases were

detected in *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2, indicating that the FHL complex of SB1 and SB2 consists of a group 4 [NiFe]-hydrogenases.

Other FHL complexes may consist of a formate dehydrogenases and a [FeFe]-hydrogenase, for example the FHL complexes of the acetogen *Acetobacterium woodii* and the amino acid fermenter *Eubacterium acidaminophilum* (Graentzdoerffer *et al.* 2003, Poehlein *et al.* 2012). Group 4 [NiFe]-hydrogenase genes affiliated with *Acidobacteriaceae* and [FeFe]-hydrogenase genes affiliated *Neisseriaceae* were detected with *Carex* roots that derived from mire 2 (Hunger *et al.* 2016), indicating *Acidobacteriaceae* and *Neisseriaceae* that were detected on a mixture of mire 2-derived roots (Figure 43) were involved in H_2 formation from formate.

Citrobacter-related isolate SB1 and *Hafnia*-related isolate SB2 co-metabolized formate and glucose (Figure 44), and it is likely that formate would be utilized as a co-substrate by those taxa under *in situ* conditions due to the availability of diverse root-derived organic molecules. In addition, the formation of diverse fermentation products such as ethanol, lactate, and H_2 by *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 (Figure 44) underscore the likelihood that such FHL-containing microorganisms can be trophically linked to secondary consumers in the root-zone. In this regard, *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 derived from the initial enrichment where the acetogenic enrichment FH was obtained from. This initial enrichment already converted H_2-CO_2 and formate to predominantly acetate, indicating that *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 might have had a trophic interaction with the acetogen in the initial enrichment. Similar to trophic interactions with acetogens and fermenters mentioned above (6.4), fermentation-derived lactate and H_2-CO_2 from *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 might have been used by the *Clostridium*-related acetogen in the initial enrichment. Additionally, 16S rRNA gene sequences affiliated with *Thermodesulfovibrio yellowstonii*, *Aciditerrimonas ferrireducens*, *Clostridium amygdalinum*, *Sulfurospirillum halorespirans* that reduce sulfate, thiosulfate, nitrate, or iron(III) and use H_2 as source for

reductant have been detected on mire-derived roots (Figure 43, 6.6.2), suggesting that H_2 may partially be utilized by taxa capable of anaerobic respiration in the root-zone.

Under *in situ* conditions O_2 leaks periodically from roots (Armstrong *et al.* 1991, Kraemer and Alberte 1995), and considering the ability of *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 to respire O_2 such an interaction would be beneficial for O_2-sensitive acetogens. Formate consumption did not yield appreciable amounts of H_2 when *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2 respired O_2, illustrating that the potential of H_2-scavenging microorganisms such as acetogens, methanogens, photoheterotrophs, sulfate reducers, nitrate reducers, and iron(III) reducers to utilize formate-derived H_2 will depend on the metabolic status of the FHL-containing taxa.

Those findings point out that FHL-containing taxa occur in the roots-zone and have the potential to form formate-derived H_2. Many of those taxa are likely facultative aerobes as illustrated by the detection of FHL-containing *Citrobacter*-related isolate SB1 and *Hafnia*-related isolate SB2, both of which are facultative aerobes. However, it should be noted that obligate anaerobes such as sulfate reducers and methanogens may form H_2 from formate independent of FHL by the combined activities of formate dehydrogenase and different hydrogenases (e.g., in the case of methanogens, by H_2-forming methylene tetrahydromethanopterin dehydrogenase) (Wu *et al.* 1993, Lupa *et al.* 2008, Martins and Pereira 2013, Martins *et al.* 2015). Thus, H_2 might also be produced from formate independent of FHL.

6.3 Acetogenesis and associated *Bacteria*

6.3.1 Contrasting mire ‘soils’

Acetogenesis is a known process in mire ‘soils’ but active acetogens are for most parts unidentified (Bräuer *et al.* 2004, Deppe *et al.* 2010, Hunger *et al.* 2011a). About 22-67 % and 12-43 % of H_2-derived reductant was recovered in CH_4 and acetate (Table 26, Figure 19), respectively, indicating that acetogenesis competed with methanogenesis for H_2-CO_2 in

slurries of all mire 'soils'. The apparent production of CH_4 from H_2-CO_2 occurred before the apparent H_2-CO_2-dependent production of acetate in 'soil' slurries from mires 2-4 (Figure 19), whereas the apparent production of acetate from H_2-CO_2 occurred before the apparent H_2-CO_2-dependent production of CH_4 in 'soil' slurries of mire 1. Calculations of Gibbs free energy indicated that methanogenesis was thermodynamically more favorable than acetogenesis in 'soil' slurries from acidic mires 2-4, whereas acetogenesis was thermodynamically more favorable than methanogenesis in 'soil' slurries from pH-neutral mire 1, indicating that methanogenesis outcompeted acetogenesis in acidic 'soil' slurries to a certain extend and vice versa in neutral 'soil' slurries. In this regard, bog 'soil' methanogens and acetogens can compete for H_2-CO_2 until approximately four millimolar acetate is produced, which is likely due to the acetate-dependent impairment of methanogenesis (Bräuer *et al.* 2004). 16S rRNA gene sequences affiliated with taxa that contain acetogens were detected in slurries of all mire 'soils' (e.g., *Clostridiaceae* [Wiegel 2009], *Holophagaceae* [Thrash and Coates 2011], *Peptococcaceae* [Ezaki 2009], *Ruminococcaceae* [Bernalier *et al.* 1996b, Rainey 2009a], *Spirochaetaceae* [Paster 2011], and *Veillonellaceae* [Rainey 2009b]). In addition, *Clostridiaceae*-affiliated sequences and increased in relative abundance due to supplemental glucose in 'soil' slurries from mire 2 and were related to the acetogens *Clostridium carboxidivorans* (98 % sequence similarity, FR733710), *Clostridium drakei* (97 % sequence similarity, Y18813), and *Clostridium magnum* (96 % sequence similarity, X77835). These acetogens grow on sugars, alcohols, and organic acids, and *C. carboxidivorans* and *C. drakei* are also capable of growth on H_2-CO_2, CO, and amino acids (Drake *et al.* 2008, Wiegel 2009). *Veillonellaceae*-affiliated sequences increased in relative abundance due to supplemental H_2-CO_2 in slurries with 'soil' from mires 2 and 4 (Table 30), and some of those sequences were related to the sequence of the acetogen *Acetonema longum* (91 % 16S rRNA sequence similarity, AJ010964) and multiple species of *Sporomusa* (89 % 16S rRNA sequence similarity; e.g., AJ279800 and Y09976). *Sporomusa* utilizes H_2-CO_2, organic acids, and alcohols whereas *Acetonema* utilizes H_2-CO_2 and sugars (Rainey 2009b). *Peptococcaceae*-affiliated sequences were related to species of *Desulfosporosinus*. Members of *Desulfosporosinus* are

obligate anaerobes that produce predominantly acetate from organic compounds and H_2-CO_2 (Ramamoorthy *et al.* 2006, Ezaki 2009). *Holophagaceae*-affiliated sequences were related to *Holophaga foetida*. *H. foetida* is an obligate anaerobe that produces acetate from aromatic compounds such as ferulate and 5-hydroxyvanillate and pyruvate (Liesack *et al.* 1994, Thrash and Coates 2011). Collectively, those acetogens are capable of utilizing diverse substrates including H_2-CO_2, organic acids, alcohols, aromatic compounds, amino acids, and sugars (Bernalier *et al.* 1996a, Drake *et al.* 2008, Ezaki 2009, Paster 2011, Thrash and Coates 2011). The broad substrate spectrum of acetogens indicates that detected acetogens not only compete with methanogens for H_2-CO_2 but also compete with other microbes for a wide range of substrates in contrasting mire 'soils'.

Formate was detected transiently in slurries with 'soil' from all mires (5.2.3) and can be utilized by acetogens (Drake *et al.* 2006, Balch *et al.* 1979). Supplemental [^{13}C]formate stimulated the production of acetate in slurries with 'soil' from mire 2 (Figure 28), acetate was enriched in ^{13}C after [^{13}C]formate supplementation, and the estimated the Gibbs free energy for formate-dependent acetogenesis in 'soil' slurries was exergonic (Figure 29), suggest that acetogens from 'soil' of mire 2 are capable to utilize formate. *fhs* sequences affiliated with the acetogens *H. foetida*, *C. drakei*, and *C. carboxidivorans* (Liesack *et al.* 1994, Drake 2009, Drake *et al.* 2008) were detected in [^{13}C]formate-supplemented slurries with 'soil' from mire 2 but none of them was labeled by [^{13}C]formate-derived carbon in 16S rRNA gene analysis (Hunger *et al.* 2011a). 16S rRNA gene sequences affiliated with those acetogens have been detected in glucose-supplemented 'soil' slurries from mire 2 (Table 31), supporting the occurrence of *Clostridium*- and *Holophaga*-affiliated acetogens in 'soil' of mire 2. *C. drakei* can grow on formate but *C. carboxidivorans* and *H. foetida* cannot (Liesack *et al.* 1994, Küsel *et al.* 2000, Liou *et al.* 2005), indicating that affiliated acetogens might have dissimilated but not assimilated formate and/or might have other properties than cultured taxa.

6.3.2 The rhizosphere of mire plants

H_2-CO_2 and formate are utilized by acetogens in mire 'soils' (Bräuer *et al.* 2004, Wüst *et al.* 2009a) but the effect of those compounds on the production of acetate in the rhizosphere of mire plants is unknown. Supplemental formate in slurries with soil-free roots from *C. rostrata*, *C. nigra*, and *M. caerulea* stimulated the production of acetate directly or indirectly by formate-derived H_2 (Figure 33, Figure 34), indicating that some mire-derived acetogens are tightly associated with plant roots. Acetogens have been detected in deep cortex cells of the roots of the sea grass *Halodule wrightii* (Küsel *et al.* 1999) and on the roots of *Spartina alterniflora* from a salt marsh (Leaphart *et al.* 2003), and have been isolated from roots of *H. wrightii* from brackish water (Küsel *et al.* 2001) and roots of *Juncus roemerianus* from a salt marsh (Gößner *et al.* 2006). In addition, acetogenesis can be associated with rice roots (Conrad and Klose 1999). The stimulation of acetate production by formate was more pronounced with soil-free roots than with root-free soils from the same patches were plant roots derived from (Figure 33, Figure 34), indicating that acetogenesis seems to play a more important role near the root than in the surrounding soil. Calculations of Gibbs free energy indicated that the production of acetate from formate was thermodynamically more favorable than from H_2, proposing that root-derived acetogens might have used formate directly.

The acetogen-containing families *Clostridiaceae*, *Holophagaceae*, *Peptococcaceae*, and *Veillonellaceae* were detected on mire-derived roots (Table 36, Figure 43). *Veillonellaceae*- and *Holophagaceae*-affiliated 16S rRNA sequences were related to non-acetogens (Figure 43). *Clostridiaceae*- and *Peptococcaceae*-affiliated 16S rRNA sequences were related to the acetogens *C. drakei*, *Clostridium scatologenes*, and *Desulfosporosinus lacus* (Figure 43). *D. lacus* (*Peptococcaceae*) produces acetate from lactate and H_2-CO_2 under the absence of alternative electron acceptors (Ramamoorthy *et al.* 2006). The relative abundance of *Clostridiaceae*-affiliated sequences (Table 36) indicated that affiliated taxa might utilize formate and H_2 as carbon and energy source. The formate- and H_2-consuming enrichment FH that was obtained from the same mixture of roots contained one acetogen that was related to the acetogens *C. drakei*, *C. scatologenes*, *C. magnum*, and *C. carboxidivorans*

(Figure 46). *C. drakei* and *C. scatologenes* are obligate anaerobes with a pH optimum of pH 5.5-7.0 (Küsel *et al.* 2000, Liou *et al.* 2005). *C. drakei*, *C. scatologenes*, and *C. carboxidivorans* utilize cellulose, sugars, alcohols, amino acids, H_2-CO_2, and H_2-CO (Küsel *et al.* 2000, Liou *et al.* 2005, Wiegel 2009). *C. magnum* grows on sugars and some alcohols but not on H_2-CO_2 (Schink 1984b). *C. drakei* and *C. scatologenes* utilize formate and lactate, and produce skatole, whereas *C. carboxidivorans* and *C. magnum* do not (Wiegel 2009, Küsel *et al.* 2000, Whitehead *et al.* 2008, Liou *et al.* 2005). The root-derived enrichment FH produced acetate from H_2, formate, lactate, and CO, and does not smell like skatole, suggesting that the acetogen in the enrichment FH is not *C. drakei*, *C. scatologenes*, *C. magnum*, or *C. carboxidivorans* but is a new species.

6.3.3 The gut of the earthworm *E. eugeniae*

Supplemental H_2 (potentially with CO_2), formate, and methanol stimulated the production of acetate in an acetogenic enrichment (Figure 50) that was obtained from gut contents of *E. eugeniae*. The production of acetate was also stimulated by H_2-CO_2 in [^{13}C]glucose-supplemented slurries with gut contents of *E. eugeniae* (Figure 51), indicating the presence of acetogens in the earthworm gut. A few 16S rRNA sequences affiliated with *Terrisporobacter glycolicus* were detected but not labeled in [^{13}C]glucose-H_2-supplemented slurries. The final acetogenic enrichment EE contained an acetogen that was related to *T. glycolicus* and *Terrisporobacter mayombei* and produced acetate from H_2-CO_2 and formate (5.5.7). *fhs* sequences affiliated with *T. glycolicus* were detected in similar abundances in unsupplemented and [^{13}C]glucose-supplemented slurries (Figure 57). *T. glycolicus* utilizes glucose, fructose, formate, lactate, and H_2-CO_2 (Gerritsen *et al.* 2014). *T. mayombei* grows on sugars, sugar alcohols, amino acids, and H_2-CO_2 (Kane *et al.* 1991). These findings indicate that the gut of *E. eugeniae* contains a *Terrisporobacter*-related acetogen that can grow on formate and H_2-CO_2, and should be able to grow on glucose but might not have been stimulated by the experimental conditions and thus was not labeled in [^{13}Cglucose-supplemented slurries.

Lachnospiraceae-affiliated *fhs* sequences were detected in [^{13}C]glucose-supplemented slurries and were more abundant in slurries with supplemental H_2 than in slurries lacking H_2 (Figure 57). Those sequences belong to the species-level phylotype PLT2 and were related to *Blautia hydrogenotrophica* and *Marvinbryantia formatexigens* (Figure 57), indicating that the affiliated microorganism was stimulated by H_2-CO_2. *M. formatexigens* utilizes sugars and cellulose if formate is added as co-substrate (Wolin *et al.* 2003). Glucose utilization yields equal amounts of succinate and acetate, together with lactate if formate is added in small amounts (Wolin *et al.* 2003). Acetate is the sole end product from glucose if high amounts of formate are added (Wolin *et al.* 2003). Formate alone without the supplementation of another carbohydrate is not utilized (Wolin *et al.* 2003). *B. hydrogenotrophica* is an obligate anaerobe that utilizes sugars (e.g., glucose), formate and H_2-CO_2 (Bernalier *et al.* 1996b). Acetate is the major end product from growth on glucose with ethanol and lactate being minor products (Bernalier *et al.* 1996b). The properties of *B. hydrogenotrophica* and *M. formatexigens* indicated that the affiliated gut-derived microorganism might be an acetogen that utilizes glucose, formate, and H_2-CO_2. A 16S rRNA phylotype related to *M. formatexigens* or *B. hydrogenotrophica* was not labeled with [^{13}C]glucose-derived carbon but another *Lachnospiraceae*-affiliated phylotype related to *R. peoriensis* was labeled (Figure 55). *M. formatexigens* and *B. hydrogenotrophica* were distantly related to *R. peoriensis* and shared 91 % and 92 % 16S rRNA gene similarity with *R. peoriensis*, respectively. A *fhs* sequence of *R. peoriensis* was not available and thus it is unresolved if 16S rRNA phylotype PLT7 and *fhs* phylotype PLT2 might belong to the same organism.

6.4 Trophic interactions of acetogens with fermenters

The acetogenic *Terrisporobacter*-related isolate KHa and the fermentative *Bacteroides*-related isolate KHb were obtained from a co-culture that derived from O_2-influenced Hawaiian forest ‘soil’. *Bacteroides*-related isolate KHb fermented substrates that were not used by *Terrisporobacter*-related isolate KHa and formed products that supported the growth of KHa. For example, xylan was not utilized by *Terrisporobacter*-related isolate KHa but was fermented

to ethanol and H_2 by *Bacteroides*-related isolate KHb (Figure 60). In contrast, co-cultures of *Terrisporobacter*-related isolate KHa and *Bacteroides*-related isolate KHb converted xylan to predominantly acetate together with minor amounts of ethanol and H_2, indicating that products from KHb (i.e., ethanol and H_2) were converted to acetate by KHa (Figure 61). The apparent capacity of co-cultures to convert xylan to acetate is noteworthy, given the commercial interest in using acetogens to convert plant biomass to useful chemicals (www.zeachem.com).

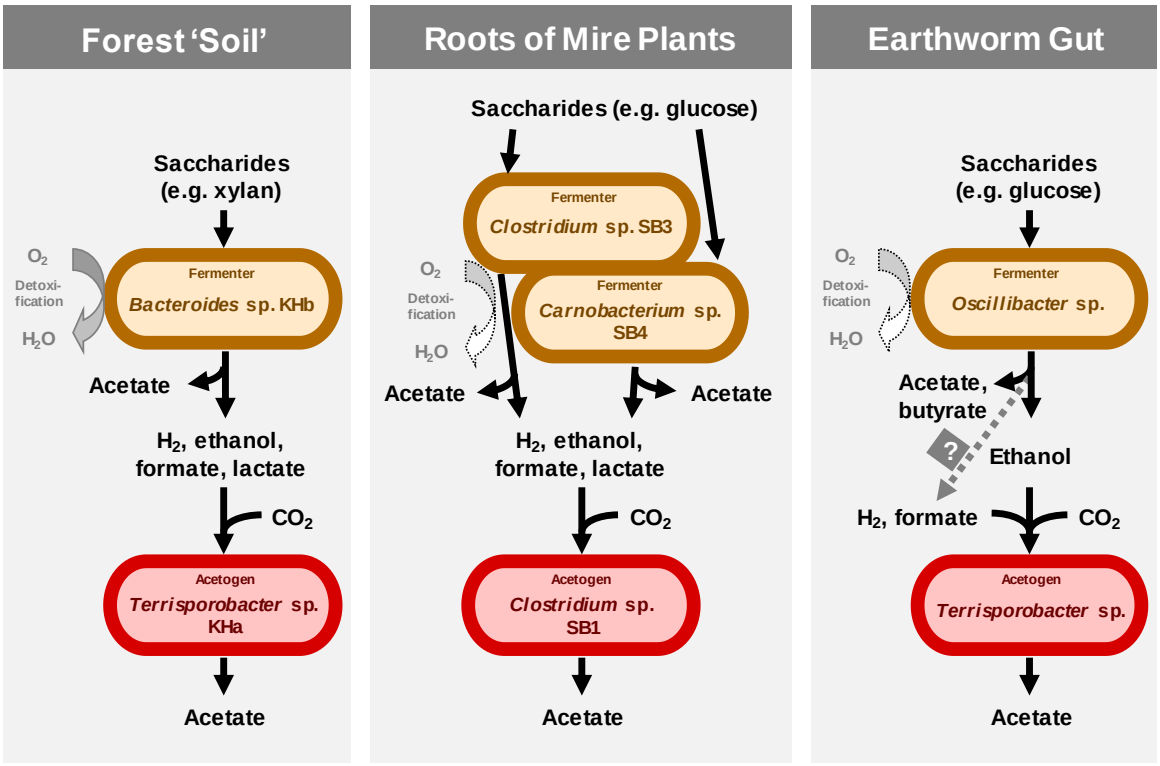


Figure 61: Hypothetical model illustrating the functional redundancy of trophic interactions between acetogens and fermenters of contrasting habitats.

Arrows with broken lines indicate processes of potential importance. The model for forest 'soil' was modified from Hunger *et al.* (2011b).

An acetogenic enrichment FH was obtained from mire-derived roots. The enrichment FH contained three microorganisms; a *Clostridium*-related acetogen, a *Clostridium*-related fermenter, and a *Carnobacterium*-related fermenter (Figure 46). The two fermenters were separated from the acetogen and were named *Clostridium*-related isolate SB3 and *Carnobacterium*-related isolate SB4. Unfortunately, attempts failed to obtain the acetogen in pure culture. *Clostridium*-related isolate SB3 and *Carnobacterium*-related isolate SB4 likely

grew on yeast extract and/or root extract (components of anoxic medium [4.4.3]) during enrichment of the acetogen. Both fermenters produced ethanol, lactate, and formate from supplemental glucose (5.4.8). *Clostridium*-related isolate SB3 also produced H_2. Acetogens of the genera *Clostridium* are capable of acetate production from ethanol, lactate, formate, and H_2-CO_2 (Küsel *et al.* 2000, Liou *et al.* 2005) and the enrichment FH produced predominantly acetate from yeast extract (included in medium) and supplemental formate and H_2-CO_2, indicating that the *Clostridium*-related acetogen produced acetate from supplemental formate and H_2-CO_2, and potentially from fermentation-derived ethanol, lactate, formate, and H_2 (Figure 61).

Another acetogenic enrichment called EE was obtained from gut contents of *E. eugeniae*. Enrichment EE contained a *Terrisporobacter*-related acetogen and a *Oscillibacter*-related fermenter (5.5.7). *Oscillibacter ruminantium* and *Oscillibacter valericigenes* are the next closest relatives of *Oscillibacter*-related fermenter that derived from the acetogenic enrichment EE. *O. ruminantium* and *O. valericigenes* are obligate anaerobes that ferment sugars such as glucose, xylose and ribose (Lee *et al.* 2013b, Iino *et al.* 2007). *O. valericigenes* can also grow on arabinose but *O. ruminantium* cannot (Lee *et al.* 2013b, Iino *et al.* 2007). *O. ruminantium* produces butyrate as major fermentation end product together with minor amounts of acetate, ethanol, and butanol (Lee *et al.* 2013b). *O. valericigenes* produces valeric acid as major fermentation end product (Iino *et al.* 2007). It is not reported if one or both species produce lactate, formate, and/or H_2 as fermentation product (Iino *et al.* 2007, Lee *et al.* 2013b). *Oscillibacter*-related fermenter likely grew on yeast extract and/or worm extract (components of anoxic medium [4.4.5]) during enrichment of the acetogen. *T. glycolicus* strain mammoth-9 was the next closest relative of *Terrisporobacter*-related acetogen. *T. glycolicus* strain mammoth-9 was isolated from the human gut (Tidjani unpublished). Some strains of *T. glycolicus* are acetogenic and predominantly produce acetate from sugars, formate and H_2-CO_2 (strain KHa [5.6.1], RD-1 [Küsel *et al.* 2001]). The utilization of butyrate is not known from strains of *T. glycolicus* (Küsel *et al.* 2001, Hunger *et al.* 2011b) or from other acetogens (Drake *et al.* 2006). Enrichment EE produced predominantly acetate together with traces of organic

acids such as butyrate and ethanol from supplemental yeast extract (included in anoxic medium [4.4.5]), formate, and H_2-CO_2, indicating that the *Terrisporobacter*-related acetogen produced acetate from supplemental formate and H_2-CO_2, and potentially from fermentation-derived products such as ethanol (Figure 61).

The production of lactate, formate, and H_2 by an aerotolerant fermentative bacterium and subsequent utilization by an acetogen has been observed with two other commensal co-cultures, namely *Thermicanus aegyptius* (fermenter) with *Moorella thermoacetica* (acetogen) (Größner *et al.* 1999) and *Clostridium intestinale* (fermenter) with *Sporomusa rhizae* (acetogen) (Größner *et al.* 2006). With *Bacteroides*-related fermenter KHb and *Terrisporobacter*-related acetogen KHa that derive from Hawaiian forest 'soil' it has been observed that ethanol ca be a functional link between an aerotolerant fermenter and an acetogen, reinforcing the likelihood of a similar trophic interaction between (a) *Carnobacterium*-related fermenter SB4, *Clostridium*-related fermenter SB3, and Clostridium-related acetogen SB1, and (b) *Oscillibacter*-related fermenter and the *Terrisporobacter*-related acetogen that derived from the gut of *E. eugeniae*.

Acetogens are classically considered to be obligate anaerobes (Drake *et al.* 2008). *Terrisporobacter*-related isolate KHa tolerated minimal amounts of O_2, a characteristic shared with other acetogens (e.g., *Sporomusa aerivorans*, *S. rhizae, T. glycolicus* RD-1) (Küsel *et al.* 2001, Karnholz *et al.* 2002, Boga and Brune 2003, Boga *et al.* 2003, Größner *et al.* 2006) whereas fermentative *Bacteroides*-related isolate KHb tolerated and consumed higher amounts of O_2 than did the acetogen KHa, a pattern also observed with the aforementioned commensal partnerships *T. aegyptius* with *M. thermoacetica* (Größner *et al.* 1999) and *C. intestinale* with *S. rhizae* (Größner *et al.* 2006). The aforementioned partnerships, the parent enrichment KH from Hawaiian forest 'soil', enrichment FH from mire-derived roots, and the enrichment EE from gut contents of *E. eugeniae* have in common that they were composed of two or three functionally linked bacteria, one being an acetogen and the other being a fermenter. Although the isolation of an acetogen together with an aerotolerant fermenter might

be considered a laboratory phenomenon, the accidental isolation and enrichment of five such partnerships illustrates a type of interaction that might occur *in situ* between so called obligate anaerobes and aerotolerant fermentative microorganisms. In the case of acetogens in habitats subject to fluctuations of O_2 availability (e.g., temporarily O_2-influenced soil, rhizosphere), it would seem beneficial to be associated with O_2-consuming aerotolerant fermentative microorganisms that convert non-acetogenic substrates to products that can subsequently support acetogenic growth.

6.5 Methanogenesis and associated *Archaea*

6.5.1 Contrasting mire 'soils'

Methanogenesis in slurries of all mire 'soils' was stimulated by fermentation-derived intermediates, supplemental acetate, and supplemental H_2-CO_2 (5.2). *mcrA* sequences that were affiliated with aceticlastic and hydrogenotrophic methanogens were detected in all mire 'soils' but with variable relative abundances (Figure 24).

"*Methanosaetaceae*"-affiliated sequences were more abundant in the near neutral pH 'soil' of mire 1 than in the other more acidic mire 'soils' 2-4 (Figure 24), suggesting that "*Methanosaetaceae*" is not well adapted to acidic conditions. This possibility is reinforced by earlier observations on the lower abundance of "*Methanosaetaceae*" in more acidic Finnish peatland 'soils' than in less acidic Finnish peatland 'soils' (Putkinen *et al.* 2009). *Methanosarcinaceae*-affiliated sequences were detected in 'soil' slurries of all mires but only in acidic 'soils' from mires 2-4 (Figure 24). Species of *Methanosaeta* (*Methanosaetaceae*) and *Methanosarcina* (*Methanosarcinaceae*) dissimilate acetate for the production of CH_4 (Garrity and Holt 2001). Species of *Methanosarcina* also grow on H_2-CO_2 and may only use acetate if H_2 is depleted (Garrity and Holt 2001). Small amounts of H_2 were produced during the degradation of acetate in 'soil' slurries of mires 1-3 (Figure 20), an observation that has been made with pure cultures of *Methanosarcina* (Garrity and Holt 2001).

Acetate stimulated the production of CH_4 (Figure 20), and *mcrA* sequences affiliated to obligate aceticlastic methanogens (i.e., species of *Methanosaeta*) were detected (Figure 24), suggesting that acetate was dissimilated via aceticlastic methanogenesis. Recovery of acetate-derived reductant with slurries from mire 1 and 2 indicated that the dissimilation of endogenous substrates was enhanced by supplemental acetate, i.e. that acetate might have had a priming effect on methanogens and potentially other microorganisms capable of utilizing endogenous substrates for dissimilation and acetate for assimilation (Fontaine *et al.* 2004). In this regard, many *mcrA* sequences that were affiliated with hydrogenotrophic methanogens were detected in slurries after the supplementation of acetate, implying that hydrogenotrophic methanogens might have been stimulated by acetate. Species of *Methanocella* (*Methanocellaceae*) and *Methanoregula* (*Methanoregulaceae*) produce CH_4 from H_2-CO_2 and cannot dissimilate acetate but require acetate for assimilation and growth (Sakai *et al.* 2008, Sakai *et al.* 2010, Bräuer *et al.* 2011, Yashiro *et al.* 2011). The priming effect was observed with slurries from mire 1 and 2 (Table 27) and *mcrA* sequences affiliated with *Methanocellaceae* were only detected in 'soils' from mire 1 and 2 (Figure 24), indicating that the priming effect was at least partially a result of the dissimilation of endogenous substrates and assimilation of supplemental acetate by *Methanocella*-related methanogens. Although *Methanocellaceae* was not detected in 'soils' of the oligotrophic acidic mires 3 and 4, *Methanocellales* has been detected in another oligotrophic acidic mire 'soil' at a depth of 25 cm or deeper (Lin *et al.* 2014a), suggesting that *Methanocellaceae* may occur in oligotrophic acidic mire 'soils' but may not always be important to methanogenesis.

Formate was detected transiently in 'soil' slurries from all mires. Some species of *Methanoregulaceae*, *Methanocellaceae*, and *Methanobacteriaceae* can utilize formate, such as *Methanoregula formicica*, *Methanolinea mesophila*, *Methanocella paludicola*, *Methanocella arvoryzae*, and *Methanobacterium formicicum* (Garrity and Holt 2001, Sakai *et al.* 2008, Sakai *et al.* 2010, Yashiro *et al.* 2011, Sakai *et al.* 2012). Species-level *mcrA* phylotypes closely related to those species were detected in all mire 'soils' (Figure 24), indicating that formate may also be a precursor of CH_4 production in those 'soils'.

Most of the detected cultured methanogens grow over a pH range of five to nine but prefer pH neutral conditions (Garrity and Holt 2001). An exception is *Methanoregula boonei* (*Methanoregulaceae*) that has a more acidic pH range (i.e., pH 4.5 to 5.5) (Bräuer *et al.* 2011). The properties of *M. boonei* are consistent with the observation that the relative abundance of *Methanoregula*-affiliated sequences increased in mire 'soils' (Figure 23) with decreasing pore water pH (Table 24). Methanogens that can dissimilate H_2-CO_2 accounted for 56-87 % of the detected abundance of methanogens in mire 'soils', whereas methanogens that can dissimilate acetate accounted for only 22-39 % of the detected abundance of methanogens, highlighting the potential contrasting importance of hydrogenotrophic and aceticlastic methanogenesis in the investigated mires.

6.5.2 The rhizosphere of mire plants

Formate is one of the most important low-molecular-weight organic acids that are released from roots of wetland plants (Koelbener *et al.* 2010), and can serve as a substrate for methanogenesis (Balch *et al.* 1979). Supplemental formate stimulated the production of CH_4 directly or indirectly due to the production of formate-derived H_2 in slurries with soil-free roots from *C. rostrata* but not with roots from *C. nigra* or *M. caerulea* (5.4). The production of CH_4 from supplemental formate was observed in all slurries with root-free soil from the same patched where roots derived from (5.4), indicating that methanogenesis may occur on mire derived-roots but is more pronounced in the surrounding soil. Methanogenesis was one of the minor processes on mire derived-roots in comparison to formate-driven acetogenesis and the formation of formate-derived H_2. The production of CH_4 from formate was thermodynamically more favorable than from H_2 (Figure 37), indicating that root-derived methanogens might have used formate directly. *mcrA* sequences affiliated to hydrogenotrophic and aceticlastic methanogens were obtained from soil-free roots of *C. rostrata* (Figure 40). Species of *Methanoregulaceae* and *Methanosarcinaceae* can be abundant in mire 'soil' cores that contain roots (Figure 24, Hunger *et al.* 2011a, Schmidt *et al.* 2016) and were also the most abundant families (together with *Methanobacteriaceae*) from roots prior to incubation (Figure 40). In

contrast to *Carex* roots, species of *Methanobacteriaceae* were detected with low abundances in contrasting mire 'soils' (Figure 24), indicating that species of *Methanobacteriaceae* may be more important on roots than in soils in terms of relative contribution to CH_4 production. *Methanoregulaceae*-affiliated sequences were related to *Methanoregula boonei* and *Methanosarcinaceae*-affiliated sequences were related to *Methanobacterium lacus* (Figure 40). *M. boonei* and *M. lacus* produce CH_4 from H_2 and CO_2 but not from formate (Bräuer *et al.* 2011, Borrel *et al.* 2012, Cadillo-Quiroz *et al.* 2014). Other members of *Methanoregulaceae* such as *Methanoregula formicica* can utilize H_2 and formate (Yashiro *et al.* 2011); however, the detected *mcrA* sequences were only distantly related to this taxon. The detected *Methanoregula*-affiliated sequences were most closely related to species that utilize H_2 and CO_2 (i.e., *M. boonei*, 92-98 % amino acid sequence similarity) rather than formate (i.e., *M. formicica*, 83-86 % amino acid sequence similarity). Acetate is not utilized for the production of CH_4 by *M. boonei* and *M. lacus* but stimulates the growth of these species (Bräuer *et al.* 2011, Borrel *et al.* 2012, Cadillo-Quiroz *et al.* 2014). Unlike other species of *Methanosarcina* that can grow on H_2 (Balch *et al.* 1979), *Methanosarcina horonobensis* grows on acetate but not on H_2 or formate (Shimizu *et al.* 2011). In this regard, the highest relative abundance of *M. horonobensis*-affiliated sequences was detected in formate-supplemented *Carex* root incubations in which acetate was readily available (Figure 33, Figure 40). *Methanosaeta*-affiliated sequences were only detected with *Carex* roots before incubation (Figure 40). That members of this acetate-utilizing genus grow slower than acetate-utilizing species of the genus *Methanosarcina* (Jetten *et al.* 1992) is consistent with the occurrence of *Methanosarcina*-affiliated taxa rather than *Methanosaeta*-affiliated taxa after prolonged incubations in which acetate was readily available. Based on the relative abundance of sequences detected with *Carex* roots (Figure 40), methanogens related to *Methanocella paludicola* and *Methanocella arvoryzae* had a low relative abundance on *Carex* roots. *M. paludicola* and *M. arvoryzae* use formate, H_2, and CO_2 but not acetate for methanogenesis and occurred in low abundance in mire 'soil' cores containing roots (Figure 24, Sakai *et al.* 2008). *Methanocella*-related taxa associated with *Carex* roots were not stimulated by supplemental formate (Figure 40), which

is in contrast to earlier studies with 'soil' from the same mire (Hunger *et al.* 2011a) and might an indication of different methanogenic communities on roots and the surrounding soils.

Approximately 74 % of the methanogens detected on roots before the incubation were affiliated to taxa that utilize H_2 and CO_2, whereas only approximately 7 % were affiliated to methanogenic taxa that utilize formate in addition to H_2 and CO_2 (Figure 40). Even so, acetate accumulated in response to formate-pulses and a degradation was not observed, almost all detected methanogens are capable of dissimilation of acetate or require acetate for assimilation, indicating an essential importance of acetate to root-derived methanogens. In earlier studies with 'soil' from the same mire, microorganisms related to *Methanobacterium formicicum* and *Methanocella* species were labeled from [^{13}C]formate-derived carbon (Hunger *et al.* 2011a). Those taxa have not been detected or not been stimulated by supplemental formate in slurries with *Carex* roots, indicating that formate-dependent methanogenesis might be driven by different methanogens on roots and surrounding soil. The collective observations on methanogenic activities and detected methanogenic taxa suggest that H_2-dependent methanogenesis was more important than formate-dependent methanogenesis to the initial methanogenic activity associated with *Carex* roots.

6.5.3 The gut of the earthworm *E. eugeniae*

Earthworms are known to emit the greenhouse gas N_2O (Horn *et al.* 2006a). Recently, it has been observed that earthworms of the species *E. eugeniae* also emit the CH_4 *in vivo* (Depkat-Jakob *et al.* 2012). [^{13}C]glucose-supplemented slurries with gut contents of *E. eugeniae* produced ^{13}C-enriched CH_4 (Table 41), indicating that CH_4 emitted from earthworms derives from methanogenesis in the earthworm gut. Supplemental [^{13}C]glucose stimulated the production of H_2, CO_2, acetate, and formate that were potentially used for the production of CH_4. *mcrA* sequences and transcripts affiliated with hydrogenotrophic and aceticlastic methanogens have been detected in gut contents of *E. eugeniae* (Figure 48). All species-level phylotypes that were detected in gut contents were also detected in the substrate used to raise earthworms on (Figure 49), indicating that gut-associated methanogens derived

from the substrate earthworms were raised on. Relative abundances of taxa detected in gut contents and substrate were similar for *mcrA* and *mcrA* transcript sequences, except for species-level phylotype PLT11 (Figure 49).

Species-level phylotype PLT11 is related to *Methanobacterium formicicum* (*Methanobacteriaceae*) and became activated in gut contents. In this regard, *M. formicicum*-affiliated methanogens were also detected in the formate- and H_2-utilizing methanogenic enrichment that derived from gut contents of *E. eugeniae* (Table 27) and were labeled with [^{13}C]glucose-derived carbon in an earlier study with gut contents of *E. eugeniae* (Schulz *et al.* 2015). *M. formicicum* uses H_2-CO_2 and formate for the production of CH_4 (Balch *et al.* 1979). *mcrA* transcripts affiliated with *Methanobacterium ivanovii* (*Methanobacteriaceae*) were detected (Figure 49). *M. ivanovii* utilizes H_2-CO_2 for the production of CH_4 and requires acetate for growth (Garrity and Holt 2001). This results indicates, that *Methanobacteriaceae*-affiliated methanogens may grow on formate and H_2 in the gut of *E. eugeniae*.

Methanosarcinaceae-affiliated sequences were related to *Methanosarcina mazei* and formed the most abundant phylotype on both gene and transcript level in gut contents as well as in the substrate (Figure 49). *M. mazei* uses H_2-CO_2, acetate, methanol, methylamine and trimethylamine for the production of CH_4 (Liu *et al.* 1985). Growth on acetate can be very weak with some strains and very rapidly with other strains (Liu *et al.* 1985), and acetate may only be used when H_2 is not available (Garrity and Holt 2001). Unsupplemented gut contents showed an immediate release of H_2 (Figure 51), indicating that H_2 is also present in the gut of *E. eugeniae* and proposing that *Methanosarcina*-affiliated phylotypes dissimilated likely H_2 rather than acetate.

Methanomicrobiaceae-affiliated sequences were more often detected than *Methanomicrobiaceae*-affiliated transcripts in gut contents, and were related to *Methanoculleus palmolei*, *Methanoculleus chikugoensis*, and *Methanoculleus marisnigri* (Figure 49). *M. palmolei*, *M. chikugoensis*, and *M. marisnigri* produce CH_4 from H_2-CO_2, formate, 2-propanol-CO_2, 2-butanol-CO_2, and cyclopentanol-CO_2 (Maestrojuán *et al.* 1990,

Zellner *et al.* 1998, Dianou *et al.* 2001), indicating that some methanogens in the gut of *E. eugeniae* might grow on secondary alcohols. *M. palmolei* and *M. chikugoensis* require acetate for assimilation but *M. marisnigri* does not (Romesser *et al.* 1979, Maestrojuán *et al.* 1990, Zellner *et al.* 1998, Dianou *et al.* 2001).

mcrA sequences or transcripts affiliated with *Methanoregula formicica*, *Methanospirillum hungatei*, *Methanocella paludicola*, *Methanobacterium palustre*, and *Methanosaeta concilii* were rarely detected, indicating that related phylotypes were of minor importance in gut contents of *E. eugeniae*. *M. formicica*, *M. hungatei*, *M. paludicola*, and *M. palustre* are hydrogenotrophic methanogens that utilize H_2-CO_2 or formate (Ferry *et al.* 1974, Sakai *et al.* 2008, Yashiro *et al.* 2011), whereas aceticlastic *M. concilii* uses only acetate for the production of CH_4 (Touzel *et al.* 1988, Patel and Sprott 1990). *M. palustre* was isolated from a peat bog and can also use propanol-CO_2 for the production of CH_4 (Zellner *et al.* 1989).

Methanogens that can dissimilate H_2-CO_2 accounted for 96-99 % in gut contents, whereas methanogens that can dissimilate acetate and H_2-CO_2 accounted for only 55-65 %, highlighting the potential importance of hydrogenotrophic and aceticlastic methanogenesis in gut contents of *E. eugeniae*. About 25 % of detected *mcrA* phylotypes were affiliated with methanogens that use secondary alcohols such as 2-butanol and 2-propanol together with CO_2 in addition to H_2-CO_2 for the production of CH_4, indicating that methanogenesis in gut contents of *E. eugeniae* might also be driven by secondary alcohols. In this regard, taxa that produce secondary alcohols were detected in slurries with gut contents, for example *Oscillibacter ruminantium* produces butanol from glucose fermentation (Lee *et al.* 2013b).

6.6 Other processes and associated *Bacteria*

6.6.1 Contrasting mire ‘soils’

Sulfate and/or nitrate were detected in all mire ‘soils’ (Table 24). 16S rRNA gene sequences that were affiliated with taxa capable of anaerobic respiration were detected in mire ‘soils’ and/or ‘soil’ slurries (e.g., *Acidimicrobiaceae*, *Bacillaceae*, *Bradyrhizobiaceae*,

Comamonadaceae, *Conexibacteraceae*, *Desulfobacteraceae*, *Geobacteraceae*, *Holophagaceae*, *Hyphomicrobiaceae*, *Moraxellaceae*, *Mycobacteriaceae*, *Neisseriaceae*, "*Nitrospiraceae*", *Rhodospirillaceae*, *Steroidobacter*, *Thermomonosporaceae*) (Table 48). Collectively, the detected taxa are capable to reduce sulfate, nitrate, iron(III), and humic acids, and may utilize organic acids, multi-carbon compounds, or H_2 as sources of reductant (Coates *et al.* 1999, Kuever *et al.* 2005, Juni and Bøvre 2005). Most of those taxa are aerobes that preferentially respire different carbohydrates such as sugars, organic acids, amino acids, or alcohols (e.g., *Acidimicrobiaceae* [Itoh *et al.* 2011], *Bacillaceae* [Logan and De Vos 2009], *Bradyrhizobiaceae* [Garrity *et al.* 2005e], *Comamonadaceae* [Spring *et al.* 2004], *Conexibacteraceae* [Whitman and Suzuki 2012], *Hyphomicrobiaceae* [Garrity *et al.* 2005f], *Methylocystaceae* [Xie and Yokota 2005], *Moraxellaceae* [Pagnier *et al.* 2011], *Mycobacteriaceae* [Magee and Ward 2012], *Neisseriaceae* [Lin *et al.* 2008, Lee *et al.* 2013a], *Rhodospirillaceae* [Dziuba *et al.* 2016], *Steroidobacter* [Fahrbach *et al.* 2008, Sakai *et al.* 2014], *Thermomonosporaceae* [Goodfellow and Trujillo 2012]), and some grow via phototrophy (e.g., *Bradyrhizobiaceae* [Garrity *et al.* 2005e], *Hyphomicrobiaceae* [Garrity *et al.* 2005f]), or methylotrophy (e.g., *Hyphomicrobiaceae* [Garrity *et al.* 2005f]).

Acidimicrobiaceae, *Bradyrhizobiaceae*, and a cluster related to *Conexibacteraceae*, *Patulibacteraceae*, and *Solirubrobacteraceae* were detected in 'soils' and/or 'soil' slurries of all mires (Table 31). *Acidimicrobiaceae*-affiliated sequences were related to *Aciditerrimonas ferrireducens*. *A. ferrireducens* is an acidophilic facultative aerobe that grows on sugars under oxic conditions and reduce iron(III) autotrophically with H_2 under anoxic conditions (Itoh *et al.* 2011). Members of *Bradyrhizobiaceae* utilize nitrate as terminal electron acceptor under anoxic conditions together with sugars or amino acids (Garrity *et al.* 2005e). Growth via respiration of O_2 or photoheterotrophically is also possible (Garrity *et al.* 2005e). Most species of *Bradyrhizobiaceae* fix N_2 as intracellular nitrogen-fixing symbionts located in plant roots (Garrity *et al.* 2005e). A cluster of sequences was equally close related to 16S rRNA gene sequences of *Conexibacter*, *Patulibacter*, *Solirubrobacter* (*Conexibacteraceae*, *Patulibacteraceae*, *Solirubrobacteraceae*). Members of *Conexibacter* reduce nitrate but prefer to

reduce O_2 (Whitman and Suzuki 2012). Members of *Conexibacter*, *Patulibacter*, *Solirubrobacter* are aerobes that grow on sugars such as glucose, cellobiose or amino acids (Whitman and Suzuki 2012).

Hyphomicrobiaceae and *Thermomonosporaceae* were detected in 'soils' and/or 'soil' slurries of mires 1-3 (Table 48). *Hyphomicrobiaceae*-affiliated sequences were related to species of *Hyphomicrobium*, *Rhodoplanes*, or *Blastochloris*. Some species of *Hyphomicrobium* grow anaerobically with nitrate as the terminal electron acceptor together with C_1 compounds such as methanol, methylamines, dichloromethane or methyl sulfate (Garrity *et al.* 2005f) whereas most species grow aerobically with O_2 as the terminal electron acceptor together with C_1 or C_2 compounds such as methanol and acetate (Garrity *et al.* 2005f). Species of *Rhodoplanes* are anaerobes and species of *Blastochloris* are aerobes that grow photoheterotrophically on simple organic compounds such as acetate (Garrity *et al.* 2005f). The relative abundance of *Hyphomicrobiaceae* increased in slurries with 'soil' from mire 1 due to the supplementation of glucose and acetate, indicating that affiliated microorganisms might have grown on acetate (available in high amounts in glucose-supplemented slurries) during times of exposure to light (e.g., taking samples for chemical analysis). *Thermomono-sporaceae*-affiliated sequences were related to *Actinomadura* and *Actinoallomurus*. Members of *Actinomadura* reduce nitrate but prefer O_2 as electron acceptor and grow on amino acids and sugars such as glucose (Goodfellow and Trujillo 2012). Some species of *Actinoallomurus* were isolated from roots (Indananda *et al.* 2011, Tang *et al.* 2013) and oxidize sugars or amino acids under oxic conditions (Indananda *et al.* 2011, Tang *et al.* 2013).

Holophagaceae-affiliated sequences were detected in 'soil' slurries from mires 2 and 'soils' from mire 3, and were related to *Geothrix fermentans. G. fermentans* is an obligate anaerobe that reduces iron(III) and oxidizes organic acids such as acetate (Coates *et al.* 1999, Thrash and Coates 2011).

Neisseriaceae-affiliated sequences were detected in 'soil' slurries of mires 2 and 4 (Table 48), and some sequences were related to *Pseudogulbenkiania*. Members of

Pseudogulbenkiania reduce nitrate under anoxic conditions but prefer O_2 as electron acceptor and grow on sugars, organic acids and alcohols (Lin *et al.* 2008, Lee *et al.* 2013a).

Geobacteraceae and *Moraxellaceae* were detected in 'soils' and/or 'soil' slurries of mires 1 and 2 (Table 48). *Geobacteraceae*-affiliated sequences were related to members of *Geobacter*. Members of *Geobacter* are obligate anaerobes that reduces iron(III), nitrate, and humic acids and use H_2, formate, acetate and other multi-carbon compounds as electron and/or carbon sources (Kuever *et al.* 2005). *Moraxellaceae*-affiliated sequences were related to members of *Reyranella* and *Enhydrobacter*. Members of both genera are aerobes that are capable of reducing nitrate under microaerophilic or anoxic conditions (Juni and Bøvre 2005). With O_2 present, members of *Reyranella* oxidize sugars and polymers, and grows under low pH conditions (Lee *et al.* 2014b, Kim *et al.* 2013, Pagnier *et al.* 2011), whereas members of *Enhydrobacter* oxidize ethanol and organic acids such as acetate, ethanol, lactate, succinate, and formate (Juni and Bøvre 2005).

"*Nitrospiraceae*"-affiliated sequences were detected in 'soils' and/or 'soil' slurries of mires 1 and 3 (Table 48). Detected sequences were related to *Nitrospira marina* and *Nitrospira moscoviensis*. *Nitrospira moscoviensis* uses nitrate as electron acceptor under anoxic conditions but prefers O_2 (Ehrich *et al.* 1995). *N. marina* and *N. moscoviensis* oxidize nitrite to nitrate and may use CO_2 as sole carbon source under oxic conditions (Ehrich *et al.* 1995, Watson *et al.* 1986).

Rhodospirillaceae-affiliated sequences were detected in 'soils' and/or 'soil' slurries of mires 1 and 4 (Table 48), and were related to species of *Magnetospirillum*. Members of *Magnetospirillum* prefer microaerophilic conditions and O_2 as terminal acceptor but may also use other electron acceptor such as nitrate and grow on organic acids such as butyrate and lactate (Schleifer *et al*. 1991, Dziuba *et al.* 2016).

Bacillaceae, *Comamonadaceae*, *Desulfobacteraceae*, and *Steroidobacter* (family-level phylotype 29) were detected only in 'soil' and/or 'soil' slurries of mire 1 (Table 48). The *Bacillaceae*-affiliated sequence was related to species of *Bacillus* (Table 48). Members of

Bacillus may reduce nitrate besides fermentation under anoxic conditions (Logan and De Vos 2009). Organic acids, sugars, H_2, amino acids, and alcohols support grow via respiration (Logan and De Vos 2009). *Comamonadaceae*-affiliated sequences were related to *Ottowia thiooxydans* and *Ramlibacter solisilvae*. *O. thiooxydans* reduces nitrate but prefers O_2 for respiration (Spring *et al.* 2004). *O. thiooxydans* and *R. solisilvae* are facultative aerobes (Spring *et al.* 2004, Willems and Gillis 2005, Lee *et al.* 2014a). *O. thiooxydans* grows on organic acids or amino acids (Spring *et al.* 2004) whereas *R. solisilvae* grows on a few sugars (Lee *et al.* 2014a). *Desulfobacteraceae*-affiliated sequences were related to *Desulfonema magnum*. *D. magnum* is an obligate anaerobe that reduces sulfate and uses organic acids such as formate, succinate, and acetate as electron and carbon source (Kuever *et al.* 2005). The genus *Steroidobacter* belongs to the order *Xanthomonadales* but is not classified on family level (according to 'List of prokaryotic names with standing nomenclature', www.bacterio.net). Members of *Steroidobacter* reduce nitrate under anoxic conditions but prefer O_2 as electron acceptor and use steroidal hormones, polymers, sugars or acetate as electron and carbon source (Fahrbach *et al.* 2008, Sakai *et al.* 2014).

The *Mycobacteriaceae*-affiliated sequence was detected in 'soil' slurries of mire 2 (Table 48). Members of *Mycobacteriaceae* prefer O_2 as terminal electron acceptor but use nitrate as well and grow on pyruvate, citrate, and sugars such as xylose and sucrose (Magee and Ward 2012).

Some *Methylocystaceae*-affiliated sequences were related to species of *Pleomorphomonas* and increased in relative abundance due to acetate supplementation in slurries with 'soil' from mire 4 (i.e., 41 % relative abundance, Table 48). Species of *Pleomorphomonas* can grow on acetate and reduce nitrate (Xie and Yokota 2005, Madhaiyan *et al.* 2013), indicating a similar metabolism of *Pleomorphomonas*-affiliated microorganisms in mire 'soil'.

As indicated above many of the detected phylotypes are facultative aerobes. More 16S rRNA gene sequences that were detected are related to taxa that use O_2 as electron acceptor and grow on polymers, sugars, or alcohols (e.g., *Armatimonadaceae* [Tamaki *et al.* 2011], *Caulobacteraceae* [Garrity *et al.* 2005c], *Ktedonobacteraceae* [Cavaletti *et al.* 2006], *Phaselicystidaceae* [Garcia *et al.* 2009], *Polyangiaceae* [Reichenbach *et al.* 2006], *Rhodocyclaceae* [Weon *et al.* 2008], *Xanthobacteraceae* [Garrity *et al.* 2005a]). *Armatimonadaceae*-affiliated sequences were detected in 'soil' slurries of mire 4 (Table 48) and were related to *Armatimonas rosea*. *A. rosea* grows on a few sugars, yeast extract, pectin and gellan gum (Tamaki *et al.* 2011). *Ktedonobacteraceae*-affiliated 16S rRNA gene sequences were detected in 'soil' slurries of mire 2 (Table 48) and were related to *Ktedonobacter racemifer*. *K. racemifer* grows under microaerophilic conditions and hydrolyzes starch (Cavaletti *et al.* 2006). A few sequences were related to *Phaselicystis flava* (*Phaselicystidaceae*) and *Byssovorax cruenta* (*Polyangiaceae*). *Phaselicystis flava* utilizes sugars (Garcia *et al*. 2009). *Byssovorax cruenta* utilizes sugars and polymers such as cellulose and chitin (Reichenbach *et al.* 2006). *Rhodocyclaceae*-affiliated sequences were detected in 'soil' slurries of mire 2 (Table 48) and were related to *Uliginosibacterium gangwonense*. *U. gangwonense* was isolated from a Korean wetland, degrades polymers such as starch and cellulose, and grows on sugars such as glucose (Weon *et al.* 2008). The *Xanthobacteraceae*-affiliated sequence was detected in 'soil' of mire 1 (Table 48) and was related to species of *Labrys* (Garrity *et al.* 2005a). Members of *Labrys* grow on sugars such as glucose (Garrity *et al.* 2005a). Family-level phylotype 1-affiliated sequences were detected in 'soils' of mires 2-4 and in 'soil' slurries of all mires (Table 48), and were related to the aerobic taxa "*Candidatus* Solibacter usitatus" and *Bryobacter aggregatus*. "*Candidatus* S. usitatus" may be able to utilize polymers, sugars, amino acids, and alcohols, whereas *B. aggregatus* utilizes polysaccharides and sugars (Ward *et al.* 2009, Kulichevskaya *et al.* 2010), suggesting that members of family-level phylotype 1 might hydrolyze polymers and oxidize sugars in mire 'soils'.

Some members of the taxa mentioned above and other detected taxa that were detected can (a) fix N_2 (e.g., *Acetobacteraceae* [Sievers and Swings 2005], *Beijerinckiaceae* [Garrity *et al.* 2005d], *Bradyrhizobiaceae* [Garrity *et al.* 2005e], *Methylocystaceae* [Bowman 2005], *Oxalobacteraceae* [Garrity *et al.* 2005g], *Rhizobiaceae* [Kuykendall 2005]), (b) grow under low pH conditions (e.g., *Acetobacteraceae* [Sievers and Swings 2005], *Acidimicrobiaceae* [Norris 2012], *Acidobacteriaceae* [Thrash and Coates 2011], *Beijerinckiaceae* [Garrity *et al.* 2005d], *Ktedonobacter* [Cavaletti *et al.* 2006], *Rhodocyclaceae* [Weon *et al.* 2008]), (c) grow via methylotrophy (e.g., *Beijerinckiaceae* [Garrity *et al.* 2005d], *Hyphomicrobiaceae* [Garrity *et al.* 2005f], *Methyloceanibacter* [Takeuchi *et al.* 2014], *Methylocystaceae* [Bowman 2005]), or (d) grow photoheterotrophically (e.g., *Acetobacteraceae* [Sievers and Swings 2005], *Bradyrhizobiaceae* [Garrity *et al.* 2005e], *Hyphomicrobiaceae* [Garrity *et al.* 2005f]). *Acetobacteraceae*-affiliated sequences were detected in 'soil' slurries of all mires (Table 48) and were related to *Rhodopila globiformis*. *R. globiformis* is a phototrophic purple non-sulfur bacterium that prefers to grow photoheterotrophically under anoxic conditions with for example gluconate, mannitol, ethanol or fructose (Sievers and Swings 2005). Other sugars or organic acids are assimilated in low concentrations (Sievers and Swings 2005). *Bradyrhizobiaceae*-affiliated sequences were detected in 'soils' and 'soil' slurries of all mires (Table 48). Most species of *Bradyrhizobiaceae* fix N_2 as intracellular nitrogen-fixing symbionts located in plant roots (Garrity *et al.* 2005e). A few sequences affiliated with *Methyloceanibacter caenitepidi* were detected in 'soil' slurries of mire 1 (Table 44). *M. caenitepidi* is a facultative methylotroph that utilizes methanol and methylamines, but can also oxidize acetate (Takeuchi *et al.* 2014). Some sequences that were affiliated with *Methylocystaceae* were related to species of *Methylocystis* (Bowman 2005). Members of *Methylocystis* are aerobic methylotrophs that grow only on CH_4 and methanol (Bowman 2005). *Beijerinckiaceae*-affiliated sequences were detected in 'soils' of mires 2-4 and in 'soil' slurries of all mires (Table 48) and were related to species of *Methanocella* and *Beijerinckia*. Members of *Beijerinckia* utilize sugars, organic acids, and alcohols whereas members of *Methanocella* oxidize methanol or CH_4 (Garrity *et al.* 2005d).

The availability of O_2 can fluctuate in mire 'soils' due to varying water tables, and taxa with facultative metabolic potentials have theoretical advantages over O_2-sensitive obligate anaerobes such as methanogens during such fluctuations in O_2 availability. In this regard, (a) detected bacterial 16S rRNA gene sequences were affiliated with taxa that are thought to be obligate aerobes (e.g., *Caulobacteraceae* [Garrity *et al.* 2005c] and *Thermomonosporaceae* [Goodfellow and Trujillo 2012], Table 31) and (b) tolerance to O_2 and/or the ability to grow under both oxic and anoxic conditions are properties of many of the detected taxa (e.g., *Acidimicrobiaceae* [Itoh *et al.* 2011] and *Acidobacteriaceae* [Thrash and Coates 2011, Pankratov *et al.* 2012], Table 31).

Some 16S rRNA gene sequences were affiliated with taxa that grow within eukaryotes (e.g., "*Candidatus* Xiphinematobacter" [Vandekerckhove *et al.* 2000], *Coxiellaceae* [Santos *et al.* 2003]), suggesting that certain eukaryotes in mire 'soils' might harbor endosymbiotic bacteria. "*Candidatus* Xiphinematobacter"-affiliated sequences were detected in 'soil' and 'soil' slurries of mire 1 (Table 48). Members of "*Candidatus* Xiphinematobacter" are known as endosymbionts of nematodes (Vandekerckhove *et al*. 2000). *Coxiellaceae*-affiliated sequences were detected in 'soil' slurries of mire 4 (Table 48) and were related to species of *Aquicella*. Members of *Aquicella* grow within protozoa (Santos *et al.* 2003). One 16S rRNA gene sequence was most closely related to *Micavibrio* (*Bdellovibrionaceae*), a taxon that preys as an exoparasite preferentially on bacteria of the genera *Pseudomonas* and *Xanthomonas* (Kuever *et al.* 2005). These collective properties illustrate the broad metabolic diversity of bacterial communities in mire 'soils'.

6.6.2 The rhizosphere of mire plants

16S rRNA gene sequences that were affiliated with taxa capable of anaerobic respiration were detected on mire-derived roots before and/or after anoxic incubation (i.e., *Holophagaceae* [Coates *et al.* 1999], *Lachnospiraceae* [Parshina *et al.* 2003], "*Nitrospiraceae*" [Spring *et al.* 1993, Henry *et al.* 1994], *Peptococcaceae* [Ramamoorthy *et al.* 2006],

unclassified family-level phylotype 1 [Itoh *et al*. 2011]). Collectively, the detected taxa are capable to reduce sulfate, nitrate, iron(III), sulfite, benzaldehyde, and thiosulfate, and may utilize organic acids, sugars, or H_2 as electron donor [Spring *et al.* 1993, Coates *et al.* 1999, Parshina *et al.* 2003].

Holophagaceae and "*Nitrospiraceae*" were the most abundant family-level phylotypes before incubation of mire-derived roots and accounted each for 27 % of the bacterial community (Table 36). *Geothrix fermentans* belongs to the family *Holophagaceae* (Figure 43) and respires iron(III) or nitrate together with organic acids such as acetate, propionate of lactate under anoxic conditions (Coates *et al.* 1999). "*Nitrospiraceae*"-affiliated sequences clustered with "*Candidatus* Magnetobacterium bavaricum" and *Thermodesulfovibrio yellowstonii*. "*Candidatus* M. bavaricum" is most abundant in microaerobic zones and grows by anaerobic respiration with iron(III) (Spring *et al.* 1993). *T. yellowstonii* is an obligate anaerobe and grows not only by fermentation but also by reduction of sulfate, sulfite, and thiosulfate, and oxidation of lactate, pyruvate, and H_2 whereas acetate is releases after incomplete oxidation of lactate and pyruvate (Henry *et al.* 1994). Sequences affiliated with unclassified family-level phylotype 1 were detected on mire-derived roots before incubation were related to *Aciditerrimonas ferrireducens* (Table 36, Figure 43). *A. ferrireducens* is a facultative aerobe that grows with pH 2.0-4.5 and respires iron(III) together with sugars or H_2 under anoxic conditions (Itoh *et al.* 2011). *Peptococcaceae*-affiliated sequences were detected before incubation of mire-derived roots and some sequences were related to *Desulfosporosinus lacus* (Table 36, Figure 43). *D. lacus* is an acetogen that also reduces sulfate or iron(III) if available and uses lactate, formate, or H_2 as electron donors. Lactate is incompletely oxidized to acetate (Ramamoorthy *et al.* 2006). *Lachnospiraceae*-affiliated sequences were detected after incubation of unsupplemented mire-derived roots and some sequences were related to *Clostridium amygdalinum* (Table 36, Figure 43). *C. amygdalinum* grows on a wide range of carbohydrates not only by fermentation but also by anaerobic respiration with sulfite, benzaldehyde, and thiosulfate as electron acceptor and for example H_2 as electron donor (Parshina *et al.* 2003). One *Campylobacteraceae*-affiliated sequence was

detected after anoxic incubation of unsupplemented mire-derived roots and was related to *Sulfurospirillum halorespirans* (Table 36, Figure 43). *S. halorespirans* grows not only by fermentation but also by anaerobic respiration under microaerophilic or anoxic conditions (Luijten *et al.* 2003). Nitrate serves as electron acceptors and lactate, pyruvate, H_2, or formate as electron donors (Luijten *et al.* 2003). Lactate and pyruvate are oxidized incompletely to acetate (Luijten *et al.* 2003). *Neisseriaceae*-affiliated sequences were detected after anoxic incubation of unsupplemented and formate-H_2-supplemented mire-derived roots and were related to *Paludibacterium yongneupense* (Table 36, Figure 43). *P. yongneupense* reduces nitrate but prefers O_2 (Kwon *et al.* 2008).

Sulfate is utilized by sulfate reducing bacteria such as *D. lacus* and *T. yellowstonii* as terminal electron acceptor (Henry *et al.* 1994, Ramamoorthy *et al.* 2006). Released sulfide from microbially mediated sulfate reduction acts as phytotoxin (Peck 1961, Lamers *et al.* 2013). Plants leak O_2 into the soil and thus counteract a reduced environment around the root that minimizes the production and thus the toxic effect of sulfide (Armstrong *et al.* 1991, Kraemer and Alberte 1995, Bezbaruah and Zhang 2005, Lamers *et al.* 2013). Also, sulfide oxidizing aerobes benefit from plant-released O_2 and plants benefit from microbially mediated sulfide oxidation (Friedrich *et al.* 2001, Lamers *et al.* 2013), and therefor building a symbiosis between wetland plants and sulfide oxidizing aerobes. Sulfide oxidizing microorganisms such as species of *Bacillus* and *Rhodocyclus* have not been detected on mire-derived roots but in ‘soils’ of analyzed mires (Table 31, mire 2 [Hunger *et al.* 2011a]), indicating the potential symbiosis of sulfide oxidizing aerobes and plants in mire ‘soils’.

As indicated above some of the detected phylotypes are facultative aerobes. More 16S rRNA gene sequences that were detected on mire-derived roots are related to taxa that use O_2 as electron acceptor and grow on polymers, sugars, organic acids, or alcohols (e.g., *Acetobacteraceae* [Tazato *et al.* 2012], *Chthoniobacter* [Sangwan *et al.* 2004], *Mycobacteriaceae* [Tsukamura *et al.* 1981], *Rhodospirillaceae* [Liu *et al.* 2010], *Solirubrobacteraceae* [An *et al.* 2011], *Streptomycetaceae* [Nagai *et al.* 2011], *Thermomonosporaceae*

[Iinuma *et al.* 1994]). Family-level phylotype 3, *Mycobacteriaceae*, and *Solirubrobacteraceae* were detected before incubation of mire-derived roots (Table 36). The family-level phylotype 3-affiliated sequence was most closely related to *Chthoniobacter flavus* (Figure 43). *C. flavus* grows with sugars and polymers at a pH of 4.0-7.0 (Sangwan *et al.* 2004). *Mycobacteriaceae*-affiliated sequences were related to *Mycobacterium tokaiense* (Figure 43). *M. tokaiense* utilizes sugars (e.g., glucose), organic acids (e.g., acetate, citrate), and ethanol (Tsukamura *et al.* 1981). *Solirubrobacteraceae*-affiliated sequences were related to *Solirubrobacter ginsenosidimutans* (Figure 43). *S. ginsenosidimutans* was isolated from agricultural soil and grows on arabinose (An *et al.* 2011). *Rhodospirillaceae*-affiliated sequences were detected before and after incubation of unsupplemented roots and were related to *Dongia mobilis* (Table 35, Figure 43). *D. mobilis* grows weakly on carbohydrates such as glucose or cellobiose (Liu *et al.* 2010). *Acetobacteraceae*-affiliated sequences were detected after incubation of unsupplemented and formate-H_2-supplemented roots and were related to *Gluconacetobacter asukensis* (Table 36, Figure 43). *G. asukensis* grows on fructose, glucose, and acetate (Tazato *et al.* 2012). *Streptomycetaceae* and *Thermomonosporaceae* were detected after incubation of formate-H_2-supplemented roots (Table 36). The *Streptomycetaceae*-affiliated sequence was related to *Streptomyces aomiensis* (Figure 43). *S. aomiensis* was isolated from soil and grows on sugars such as glucose and xylose (Nagai *et al.* 2011). The *Thermomonosporaceae*-affiliated sequence was related to *Actinocorallia herbida* (Figure 43). *A. herbida* hydrolysis starch and grows on sugars such as glucose (Iinuma *et al.* 1994).

Bradyrhizobiaceae-affiliated sequence were detected after anoxic incubation of mire-derived roots (Table 36) and were related to *Bradyrhizobium valentinum*. *B. valentinum* was isolated from N_2-fixing nodules of *Lupinus mariae-josephae* (Durána *et al.* 2014). Members of *Bradyrhizobium* fix N_2 and release ammonia as a nitrogen source for the plant (Brimecombe *et al.* 2001, Durána *et al.* 2014). Expression of nodulation genes can be triggered by plant-derived flavonoids in *Bradyrhizobium* (Brimecombe *et al.* 2001). The activation of nodulation genes is needed to form nodules on plant roots, a formation known from leguminous plants

(Brimecombe *et al.* 2001, Frankowski *et al.* 2015). *Bradyrhizobium* benefits from plant-derived carbon and the plant benefits from microbial-derived ammonia (Brimecombe *et al.* 2001). The occurrence of *Bradyrhizobiaceae*-affiliated microorganisms on roots of wetland plants (Chaintreuil *et al.* 2000) suggests that N_2-fixing microorganisms and plants might form a symbiotic link in mire 'soils'.

6.6.3 The gut of the earthworm *E. eugeniae*

Nitrate can be detected in the earthworm gut and the concentration of iron(II) decreases from the anterior to the posterior end of the gut, indicating that iron(III) is reduced to iron(II) by anaerobic respiration and subsequently is assimilated by microorganisms and/or the earthworm (Wüst *et al.* 2009b). Processed cow manure, the substrate of *E. eugeniae*, contains up to 60 mg nitrate kg^{-1} on a fresh weight basis (Depkat-Jakob *et al.* 2012) and provides a potential source of nitrate for anaerobic respiration. 16S rRNA sequences that were affiliated with taxa capable of anaerobic respiration were detected in gut contents of *E. eugeniae* before and/or after anoxic incubation (i.e., *Acetobacteraceae* [Jiang *et al.* 2006], *Aciditerrimonas*-related phylotype [Itoh *et al.* 2011], *Bradyrhizobiaceae* [La Scola *et al.* 2003], *Desulfovibrionaceae* [Nanninga and Gottschal 1987, Sakaguchi *et al.* 2002], *Enterobacteriaceae* [Brenner *et al.* 2005], *Hyphomicrobiaceae* [Garrity *et al.* 2005f], *Phyllobacteriaceae* [Labbé *et al.* 2004, Kim *et al.* 2009], *Ruminococcaceae* [Lee *et al.* 2013b]). Collectively, the detected taxa are capable to reduce sulfate, nitrate, and iron(III), and may utilize ethanol, organic acids, sugars, or H_2 as sources of reductant (Garrity *et al.* 2005f, Itoh *et al.* 2011, Nanninga and Gottschal 1987).

Aciditerrimonas-related phylotype, *Hyphomicrobiaceae*, and *Phyllobacteriaceae* were detected in light fractions before and after the incubation of gut contents with [^{13}C]glucose supplementation (Table 44). Some 16S rRNA sequences were related to *Aciditerrimonas ferrireducens* (*Aciditerrimonas*-related phylotype). *A. ferrireducens* is an acidophilic facultative aerobe that grows on sugars under oxic conditions and reduce iron(III) autotrophically with H_2 or heterotrophically with sugars under anoxic and low pH conditions (i.e., pH 2.0-4.5) (Itoh *et*

al. 2011). *Hyphomicrobiaceae*-affiliated sequences were related to species of *Hyphomicrobium vulgare* and *Rhodoplanes elegans*. Some species of *Hyphomicrobium* may reduce nitrate under anoxic conditions but prefers O_2 as terminal electron acceptor (Garrity *et al.* 2005f). *H. vulgare* grows on organic acids such as formate and acetate and sugars such as glucose or arabinose (Stutzer and Hartleb 1899, Garrity *et al.* 2005f). *R. elegans* is a phototrophic purple non-sulfur bacterium that is also capable of complete denitrification under anoxic conditions (Hiraishi and Ueda 1994). *Phyllobacteriaceae*-affiliated sequences were related to *Nitratireductor basaltis*, *Nitratireductor aquibiodomus*, and *Mesorhizobium huakuii*. *N. basaltis* and *N. aquibiodomus* reduce nitrate to nitrite but prefer O_2 as terminal electron acceptor (Labbé *et al.* 2004, Kim *et al.* 2009). *N. basaltis*, *N. aquibiodomus*, and *M. huakuii* use sugars as carbon and electron source (Jarvis *et al.* 1997, Labbé *et al.* 2004, Kim *et al.* 2009).

Acetobacteraceae-affiliated sequences were detected in light fractions before and after the anoxic incubations of the unsupplemented gut contents (Table 44) and were related to *Roseomonas terrae* and *Roseomonas lacus*. *R. lacus* reduces nitrate and grows on sugars such as rhamnose, galactose, and arabinose (Jiang *et al.* 2006). *R. terrae* and *R. lacus* are facultative aerobes that hydrolyze urea (Jiang *et al.* 2006, Yoon *et al.* 2007).

Desulfovibrionaceae-affiliated 16S rRNA sequences were detected in light fractions after the anoxic incubation of slurries with [^{13}C]glucose-supplemented gut contents (Table 44) and were related with *Desulfovibrio carbinolicus* and *Desulfovibrio magneticus*. *D. carbinolicus* and *D. magneticus* are obligate anaerobes that reduce sulfate (Nanninga and Gottschal 1987, Sakaguchi *et al.* 2002). *D. carbinolicus* uses various substrates such as H_2, ethanol, or propionate as electron and carbon sources whereas *D. magneticus* uses fewer substrates (e.g., pyruvate or lactate) (Nanninga and Gottschal 1987, Sakaguchi *et al.* 2002).

One *Bradyrhizobiaceae*-affiliated sequences was detected in light fractions before the incubation of gut contents (Table 44) and was related to *Bosea vestrisii*. *B. vestrisii* oxidizes

various sugars and some organic acids (La Scola *et al.* 2003). Other strains of *Bosea* reduce nitrate (La Scola *et al.* 2003).

Enterobacteriaceae-affiliated sequences were detected in light and heavy fractions before and after incubation (Table 44). Some of those sequences were related to *Erwinia persicina*, a facultative aerobe that not only grows via fermentation but may also reduce nitrate to nitrite (Brenner *et al.* 2005).

Ruminococcaceae-affiliated sequences were detected in light and heavy fractions in all treatments after the anoxic incubation and might be minorly labeled with [^{13}C]glucose-derived carbon (Table 44). Some of those sequences were related to *Oscillibacter ruminantium*, an obligate anaerobe that is not only capable of fermentation but may also respire and use sulfate and nitrate as terminal electron acceptor (Lee *et al.* 2013b).

As indicated above many of the detected phylotypes are facultative aerobes. More 16S rRNA sequences that were detected in gut contents of *E. eugeniae* are related to taxa that use O_2 as electron acceptor and grow on polymers, sugars, organic acids, amino acids, or alcohols (i.e., *Acidothermaceae* [Mohagheghi *et al.* 1986], *Bacillaceae* [La Duc *et al.* 2004], *Demequinaceae* [Finster *et al.* 2009], *Labilithrix* [Yamamoto *et al.* 2014], *Microbacteriaceae* [Shivaji *et al.* 2007], *Micromonosporaceae* [Goodfellow *et al.* 1990], *Nocardioidaceae* [Li *et al.* 2007], *Paenibacillaceae* [Uetanabaro *et al.* 2003], *Phaselicystidaceae* [Garcia *et al.* 2009], *Planctomycetaceae* [Bauld and Staley 1976, Kulichevskaya *et al.* 2009], *Polyangiaceae* [Reichenbach *et al.* 2006], *Rhodobacteraceae* [Maszenan *et al.* 1997], *Rhodospirillaceae* [Maszenan *et al.* 2005], *Solirubrobacteraceae* [Wei *et al.* 2014]).

Planctomycetaceae-affiliated sequences were detected in light and heavy fractions before and after the incubation of [^{13}C]glucose-supplemented gut contents (Table 44) and were related to *Planctomyces maris*, *Gemmata obscuriglobus*, and *Zavarzinella formosa* (Table 44). *P. maris* uses sugars and organic acids such as glucose and lactic acid (Bauld and Staley

1976). *G. obscuriglobus* and *Z. formosa* hydrolyze starch and utilize sugars such as glucose (Franzmann and Skerman 1984, Kulichevskaya *et al.* 2009). Other members of *Planctomycetaceae* such as *Schlesneria paludicola* are capable of fermenting carbohydrates (Kulichevskaya *et al.* 2007).

Paenibacillaceae-affiliated sequences were detected in light and heavy fractions after the incubation of [^{13}C]glucose-supplemented gut contents (Table 44) and were related to *Paenibacillus xinjiangensis*, *Paenibacillus agarexedens*, and *Paenibacillus residui*. *P. xinjiangensis*, *P. agarexedens*, and *P. residui* grow on sugars and alcohols (Uetanabaro *et al.* 2003, Lim *et al.* 2006, Vaz-Moreira *et al.* 2010).

Demequinaceae, *Microbacteriaceae*, *Polyangiaceae*, *Rhodospirillaceae*, and *Solirubrobacteraceae* were detected in light fractions before and after incubation of [^{13}C]glucose-supplemented gut contents (Table 44). *Demequinaceae*-affiliated sequences were related to *Demequina lutea* and *Demequina salsinemoris*. *D. lutea* and *D. salsinemoris* utilize sugars such as glucose (Finster *et al.* 2009, Matsumoto *et al.* 2010). *D. lutea* can also grow slowly under anoxic conditions (Finster *et al.* 2009). *Microbacteriaceae*-affiliated sequences were related to *Microbacterium indicum*, *Microbacterium kribbense*, and *Leifsonia poae*. *M. indicum*, *M. kribbense*, and *L. poae* utilize various sugars such as arabinose, galactose, or glucose (Evtushenko *et al.* 2000, Shivaji *et al.* 2007, Dastager *et al.* 2008). *Polyangiaceae*-affiliated sequences were related to *Sorangium cellulosum* and *Byssovorax cruenta*. *B. cruenta* grows on sugars and polymers such as maltose, arabinose, or cellulose (Reichenbach *et al.* 2006). *S. cellulosum* hydrolyzes cellulose, starch and chitin and grows on sugars such as glucose and xylan (Reichenbach 2005). *Rhodospirillaceae* affiliated sequences were related to *Defluviicoccus vanus*. *D. vanus* utilizes sugars, organic acids, and amino acids (Maszenan *et al.* 2005). Other members *Rhodospirillaceae* such as *Telmatospirillum siberiense* are capable of fermentation under anoxic condition (Sizova *et al.* 2007). *Solirubrobacteraceae*-affiliated 16S rRNA sequences were related to species of

Solirubrobacter. Members of *Solirubrobacter* utilize glucose and other sugars (Wei *et al.* 2014).

Bacillaceae, *Labilithrix*, *Micromonosporaceae*, *Nocardioidaceae*, and *Phaselicystidaceae* were detected in light fractions after the incubation [^{13}C]glucose-supplemented gut contents (Table 44). *Bacillaceae*-affiliated sequences were related to *Bacillus coahuilensis* and *Lysinibacillus odyssey*. *B. coahuilensis* grows on glycerol and citrate whereas *L. odyssey* utilizes acetate, pyruvate and some amino acids (La Duc *et al.* 2004, Cerritos *et al.* 2008). *Labilithrix luteola* grows on complex media (Yamamoto *et al.* 2014). *Micromonosporaceae*-affiliated sequences were related to *Actinoplanes digitatis* and *Actinoplanes humidus*. *A. digitatis* and *A. humidus* grow on various sugars such as arabinose or glucose (Goodfellow *et al.* 1990). *Nocardioidaceae*-affiliated sequences were related to *Nocardioides exalbidus* and *Nocardioides ganghwensis*. *N. exalbidus* and *N. ganghwensis* utilize sugars such as glucose and fructose (Li *et al.* 2007, Yi and Chun 2004). *Phaselicystidaceae*-affiliated sequences were related to *Phaselicystis flava*. *P. flava* grows on sugars such as fructose, arabinose, or mannose (Garcia *et al.* 2009).

Acidothermaceae and *Rhodobacteraceae* were detected in light fractions before and after the incubation of unsupplemented gut contents (Table 44). *Acidothermaceae*-affiliated sequences were related to *Acidothermus cellulolyticus*. *A. cellulolyticus* oxidizes various carbohydrates including glucose (Mohagheghi *et al.* 1986). *Rhodobacteraceae*-affiliated sequences were related to *Amaricoccus kaplicensis*. *A. kaplicensis* and other species within the *Amaricoccus* utilize organic acids, alcohols, amino acids, and sugars (Maszenan *et al.* 1997).

Some members of the taxa mentioned above and other taxa that were detected can (a) fix N_2 (e.g., *Bradyrhizobiaceae* [Garrity *et al.* 2005e]), (b) grow via methylotrophy (e.g., *Hyphomicrobiaceae* [Garrity *et al.* 2005f], *Methyloceanibacter* [Takeuchi *et al.* 2014], *Methylococcaceae* [Bodrossy *et al.* 1997]), or (d) grow photoheterotrophically (e.g.,

Bradyrhizobiaceae [Garrity *et al.* 2005e], *Hyphomicrobiaceae* [Hiraishi and Ueda 1994]), or (e) prey on other bacteria (e.g., *Rhizobiaceae* [Casida 1982]). *Hyphomicrobiaceae*-affiliated sequences were detected in light fractions before and after the incubation of gut contents with supplemental [^{13}C]glucose (Table 44) were related to species of *Hyphomicrobium vulgare* and *Rhodoplanes elegans*. Species of *Rhodoplanes* grow with O_2 as electron acceptor in the dark (Hiraishi and Ueda 1994). Organic acids (e.g., acetate, butyrate) are used as electron and carbon source for phototrophic growth (Hiraishi and Ueda 1994). Most species of *Hyphomicrobium* grow on one-carbon compounds such as methanol and methylamines, require CO_2 for growth, and can grow in mineral medium without the addition of other carbon sources (Garrity *et al.* 2005f). A few sequences affiliated with *Methyloceanibacter caenitepidi* were detected in light fractions before the incubation of gut contents (Table 44). *M. caenitepidi* is a facultative methylotroph that utilizes methanol and methylamines, but can also oxidize acetate (Takeuchi *et al.* 2014). *Methylococcaceae*-affiliated sequences were detected in light fractions after the incubation of [^{13}C]glucose-supplemented gut contents (Table 44) and were related to *Methylocaldum szegediense*. *M. szegediense* utilizes CH_4 as sole carbon and energy source (Bodrossy *et al.* 1997). *Rhizobiaceae*-affiliated sequences were detected in light fractions before and after the incubation of gut contents with supplemental [^{13}C]glucose (Table 44) and were related to *Ensifer adhaerens*. *E. adhaerens* is an aerobe that preys on Gram negative and Gram positive bacteria (Casida 1982). *Acidimicrobiaceae*-affiliated sequences were detected in light fractions before the incubation of gut contents (Table 44) and were related to *Ilumatobacter fluminis* and *Acidimicrobium ferrooxidans*. *I. fluminis* and *A. ferrooxidans* are aerobes (Clark and Norris 1996, Matsumoto *et al.* 2009). *A. ferrooxidans* oxidizes ferrous iron autotrophically or heterotrophically under low pH conditions (Clark and Norris 1996). These collective properties illustrate the broad metabolic diversity of bacterial communities in the gut contents of *E. eugeniae*.

6.7 Conclusions, limitations, and future perspectives

Greenhouse gases such as CH_4 contribute to global warming which, among others, is reflected in a rising global temperature, diminishing ice covers, and rising sea levels (Rogers and Whiteman 1991, Stocker *et al.* 2013, Schuur *et al.* 2015). A considerable amount of CH_4 is produced from methanogens in diverse anoxic habitats (Thauer 1988). The intent of this dissertation was to obtain insights into the methanogenic food webs of contrasting anoxic habitats including CH_4-emitting mire 'soils', rhizosphere of mire plants, and gut contents of the earthworm *E. eugeniae*.

Four contrasting mire 'soils' showed similar glucose-, acetate, and H_2-CO_2-dependent product profiles, cultivable cell numbers, and gene copy numbers, but major differences were observed between the microbial communities (Figure 62, Figure 63, Figure 64). Only the following three species-level *mcrA* phylotypes (i.e., 15 % of the 20 detected) were common to all mires: PLT2, PLT14, and PLT17 that were closely related to *Methanoregula boonei*, *Methanosarcina vacuolata*, and *Methanosaeta concilii*, respectively (Figure 24, Figure 63). Furthermore, only the following 13 family-level 16S rRNA gene phylotypes (i.e., 15 % of the 86 detected) were common to all mires: *Acetobacteraceae*, *Acidimicrobiaceae*, *Acido-bacteriaceae*, *Beijerinckiaceae*, *Bradyrhizobiaceae*, *Chitinophagaceae*, *Clostridiaceae*, *Methylocystaceae*, *Planctomycetaceae*, *Thermomonosporaceae*, a family-level phylotype affiliated with *Conexibacteraceae*, *Patulibacteraceae*, and *Solirubrobacteraceae*, and family-level phylotypes 1 and 41 (Table 31, Figure 63). Whereas *Actinobacteria*, *Chloroflexi*, and *Verrucomicrobia* displayed a relative abundance of 10 % or higher in certain mire 'soils', the relative abundance of these taxa was lower in other mire 'soils' (e.g., the relative abundance of *Actinobacteria* in 'soil' of mire 1 was 15 % and of mire 3 was 19 % but was only 2 % in 'soils' of mires 2 and 4) (Figure 26).

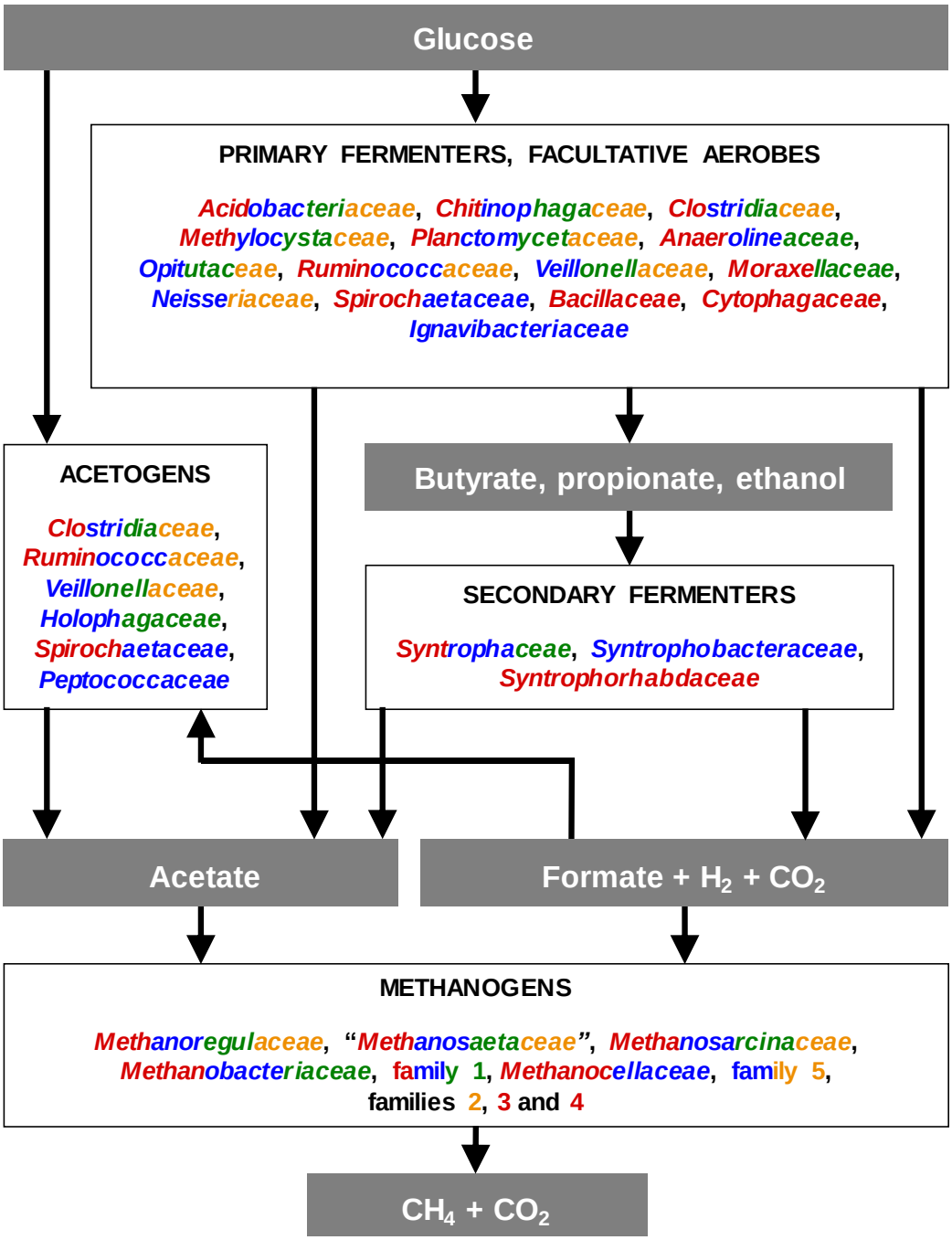


Figure 62: Hypothetical model illustrating the functional redundancy of glucose-dependent methanogenic food webs of four contrasting mire 'soils' based on detected processes and known functions of detected taxa.

Color code for phylotypes: red, mire 1; blue, mire 2; green, mire 3; orange, mire 4. *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net). Figure was modified from Hunger *et al.* (2015).

Many of the detected phyla are common taxa of mire 'soils', such as *Acidobacteria*, *Actinobacteria*, Bacteroidetes, *Chloroflexi*, *Cyanobacteria*, *Euryarchaeota*, *Firmicutes*,

Planctobacteria, *Proteobacteria*, *Spirochaetae*, and *Verrucomicrobia* (Drake *et al.* 2009, Lin *et al.* 2014a, Lin *et al.* 2014b, Dedysh *et al.* 2006, Juottonen *et al.* 2005, Kraigher *et al.* 2006, Serkebaeva *et al.* 2013, Dedysh 2011). Many of the detected family-level phylotypes of each mire 'soil' have no cultured isolates, and many of the detected phyla have been rarely reported for such 'soils' (e.g., *Armatimonadetes*, *Chlorobi*, "*Nitrospirae*") (Juottonen *et al.* 2005, Dedysh *et al.* 2006, Kraigher *et al.* 2006, Drake *et al.* 2009, Dedysh 2011, Serkebaeva *et al.* 2013, Lin *et al.* 2014a, Lin *et al.* 2014b, Schmidt *et al.* 2015). A greater number of 16S rRNA family-level phylotypes without any cultured isolates were detected in eutrophic mire 'soil' 1 (26 phylotypes) and mesotrophic mire 'soil' 2 (19 phylotypes) than in the oligotrophic mire 'soils' 3 (10 phylotypes) and 4 (11 phylotypes) (Table 31). However, the relative percentage of family-level phylotypes without any cultured isolates was similar in all mire 'soils' (i.e., 45 % for mire 1, 39 % for mire 2, 34 % for mire 3, and 37 % for mire 4 [includes both *mcrA* and 16S rRNA gene phylotypes]), emphasizing that mires contain a very significant number of uncultured bacterial taxa that await characterization.

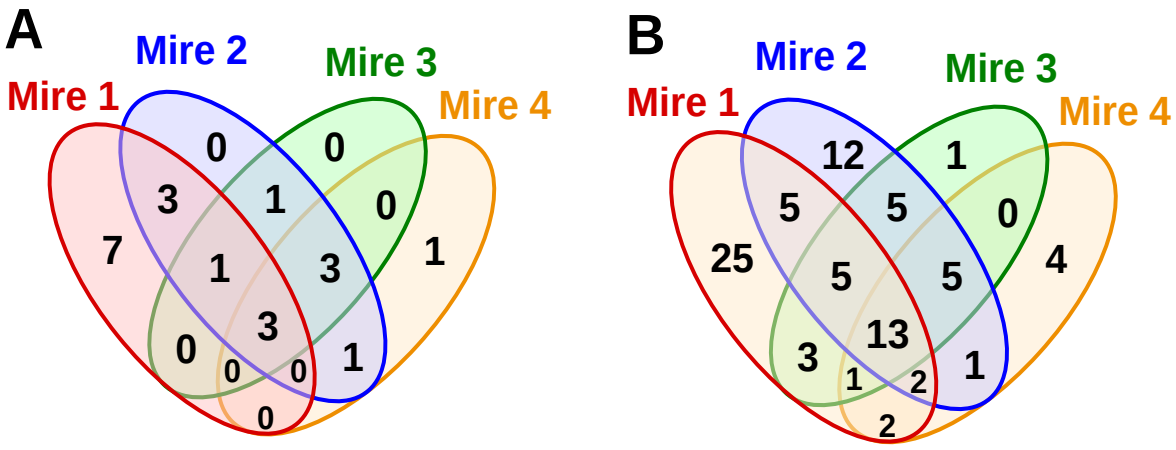


Figure 63: Venn diagram of species-level *mcrA* (A) and bacterial family-level 16S rRNA gene (B) phylotypes of contrasting mire 'soils'.

Diagram includes total number of detected phylotypes from mire 'soils' and 'soil' slurries with and without supplementation (Figure 24, Table 31). Figure was modified from Hunger *et al.* (2015).

'Soil' of mire 1 differed from other mire 'soils' mostly due to the high relative abundance of "*Methanosaetaceae*" and *Methanocellaceae*, and the neutral pH (Figure 64). 'Soil' of mire 2 differed from other mire 'soils' mostly because of the high relative abundance of novel family-level *mcrA* phylotype 1 and *Spirochaetae*. 'Soil' of mire 3 differed from other mire 'soils' mostly due to the high relative abundance of *Methanobacteriaceae* and *Methanosarcinaceae*. 'Soil' of mire 4 differed from other mire 'soils' mostly due to the high relative abundance of *Methanoregulaceae* and the high concentration of phosphate. Although it can be postulated that time of sampling may have contributed to some of the differences observed in the detected taxa, the gene copy numbers of *Archaea* and *Bacteria* in mire 'soils' may not differ significantly from one season to the next (Lin *et al.* 2014a).

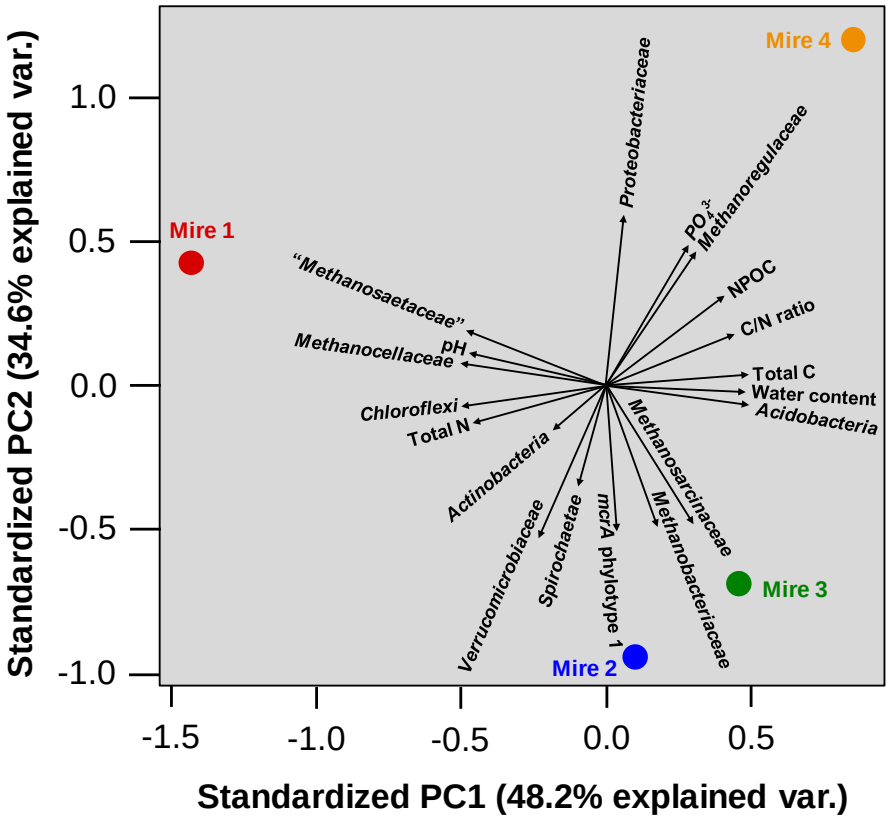


Figure 64: Principal component analysis of contrasting mire 'soils'.

Biplot was calculated with data from the following tables and figures: Table 24, Figure 23, Figure 26, Figure 27, see also 4.12.8. Due to overlapping of arrows, only parameters that were significantly different and taxa that were most abundant are displayed. Figure was modified from Hunger *et al.* (2015).

The majority of the species-level *mcrA* and family-level 16S rRNA gene phylotypes that were detected in slurries or 'soils' of mires 3 and 4 were also detected in slurries or 'soils' of mires 1 and 2 (Figure 63). The higher the water content of the mire 'soil' the higher was the C/N ratio in the 'soil', the acidity in the pore water, and the lower the diversity of detected methanogens and *Bacteria*, indicating that the mentioned factors restrict the number of dominant microorganisms in 'soils' of mires 3 and 4.

Wetland plants mediate the emission of CH_4 from soil by releasing root-derived organic carbon which serve as precursors of CH_4 production (Jones 1998, Ström *et al.* 2003). Potential substrates for methanogenesis such as formate and acetate are released from the root of wetland plants or be produced by fermentation (Koelbener *et al.* 2010, Drake *et al.* 2009), but surprisingly, methanogenesis was not a dominant process in slurries with soil-free roots from CH_4-emitting mire 2 (5.4). Soil-free roots from this mire unexpectedly produced H_2 and CO_2 as an initial response to formate, which potentially constitutes a trophic interaction between FHL-containing fermenters with acetogens and methanogens that scavenge formate-derived H_2 (Figure 65). In this regard, two isolates related to *Citrobacter* and *Hafnia* were obtained from those roots. Both isolates catalyze the formation of H_2 from formate via the formate-hydrogenlyase complex that contains a group 4 [NiFe]hydrogenase. It is worth mentioning, that similar observations were made with 'soil' from mire 2 that contained roots (6.2, Figure 28).

However, the possibility that *in situ* methanogenesis in the root-zone is periodically more significant than that initially observed under the experimental conditions of the present study cannot be excluded. Formate-derived H_2 is postulated to at least partially diffuse away from the root-zone under *in situ* conditions and become a source of energy and reductant for microbes less proximal but still very close to the root (Figure 65). In this regard, the microbial community of mire soil has diverse functional groups such as iron reducers, sulfate reducers, as well as acetogens and methanogens, that might compete for H_2 (Figure 62, 6.6.1, Paul *et*

al. 2006, Reiche *et al.* 2008, Drake *et al.* 2009), and future studies may resolve the trophic interactions between root-associated microorganisms and those proximal to the root-zone.

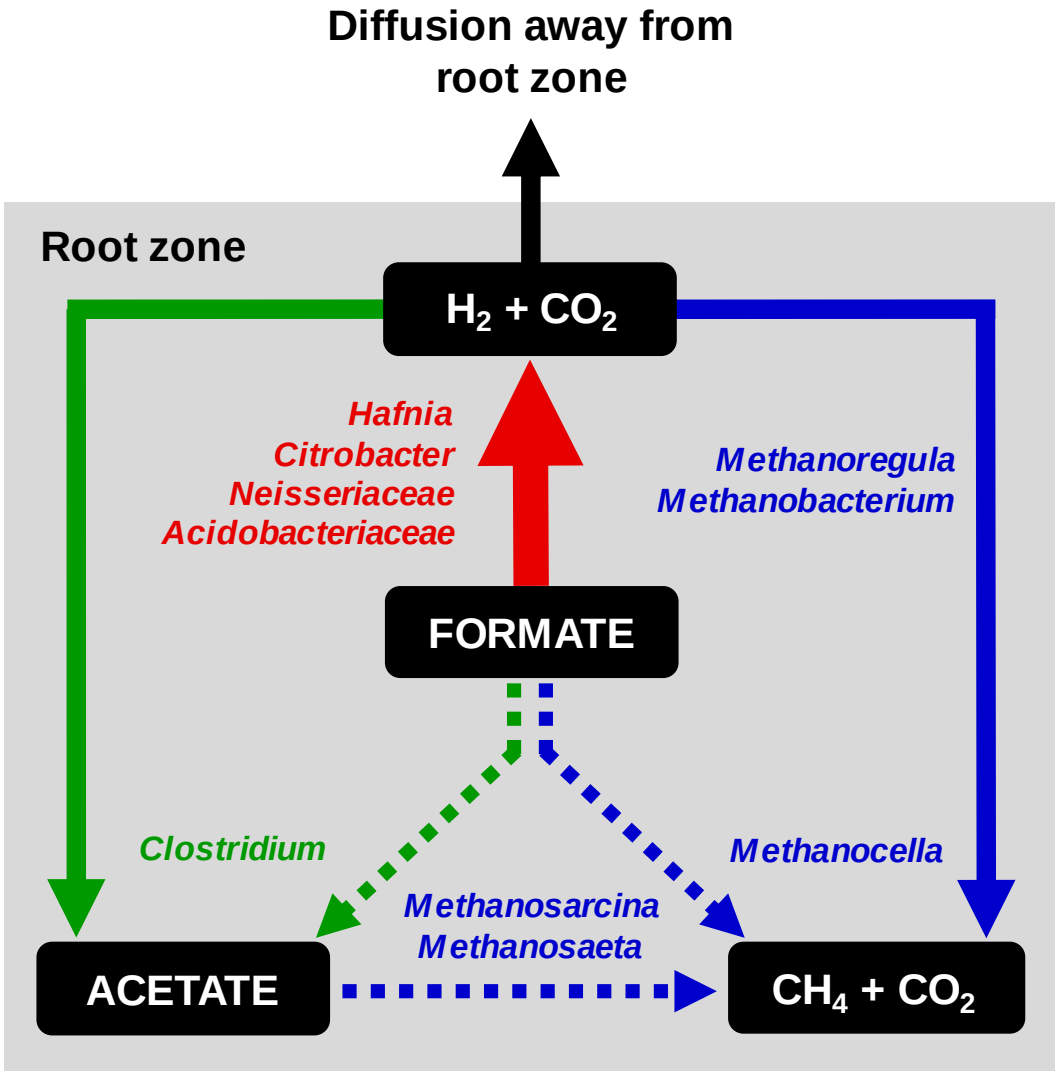


Figure 65: Hypothetical model of formate-driven anaerobic processes that can occur in association with mire roots.

Taxa are based on those detected in the present study, primarily with *Carex* roots. The dominant initial formate-dependent process detected was the transformation of formate to H_2 and CO_2, as illustrated with the large red arrow. Color code for taxa: red, FHL-containing taxa; green, acetogens; blue, methanogens. Arrows with broken lines indicate processes of potential importance. Figure was modified from Hunger *et al*. (2016).

Further insights into the methanogenic food web of the rhizosphere of mire plants could be gained by quantification and localization of taxa that were identified in contributing to the utilization of formate and H_2 on mire-derived roots (e.g., by qPCR and fluorescence microscopy). As indicated in the literature, different plants influence the emission of CH_4 differently (Ding *et al.* 2002, Kao-Kniffin *et al.* 2010, Koelbener *et al.* 2010), an observation that

can be confirmed based on the data of this study. Hitherto unknown are the factors (e.g., site, plant-specific exudates, microbial community, pH, water content) that drive the influence of plant species.

It has recently been observed that the earthworm *E. eugeniae* emits CH_4 *in vivo* (Depkat-Jakob *et al.* 2012). Supplemental glucose stimulated similar product profiles but dissimilar taxa in gut contents of the earthworm and in mire 'soils' (Figure 62, Figure 66).

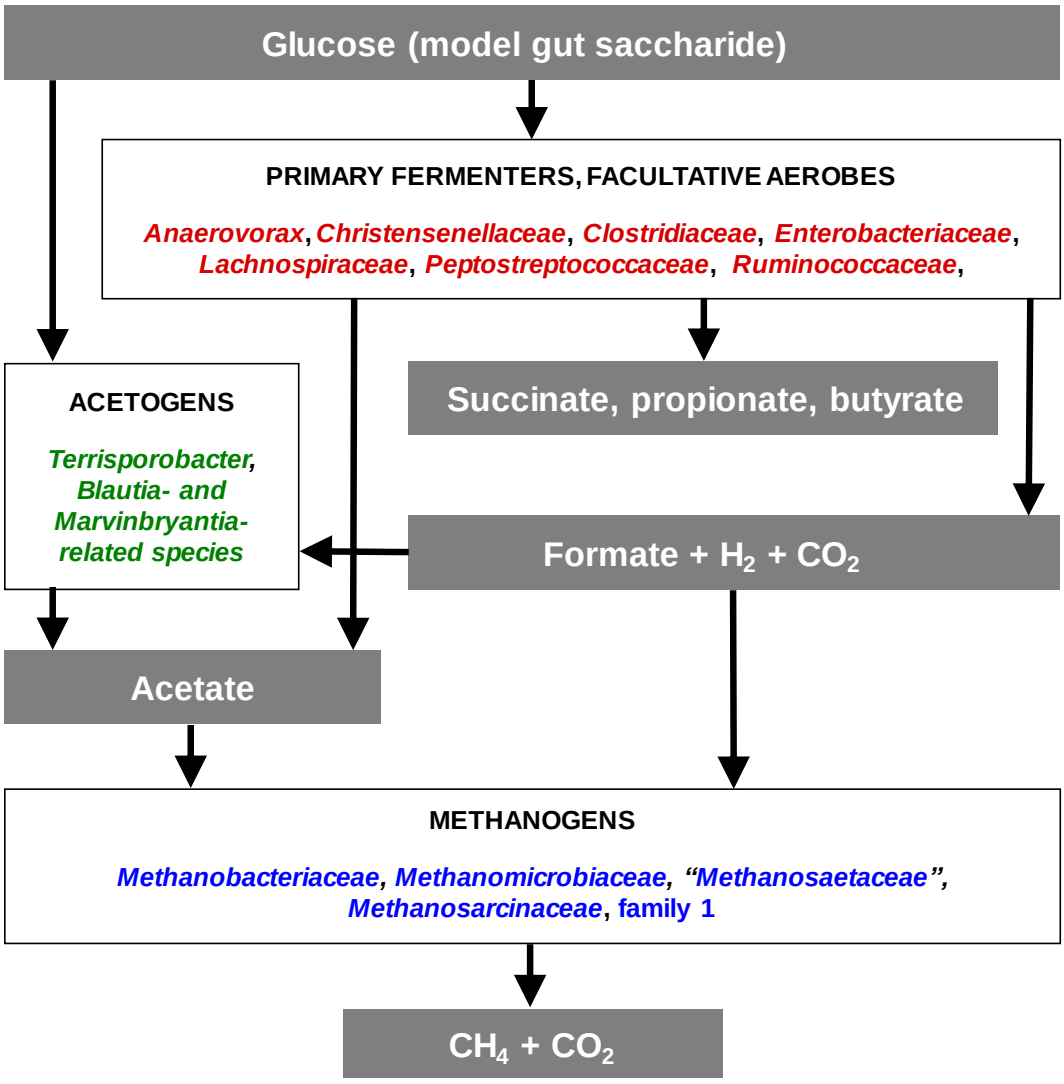


Figure 66: Hypothetical model of the glucose-dependent methanogenic food web of the earthworm gut of *E. eugeniae* based on detected processes and known functions of detected taxa.

Color code for taxa: red, fermenters; green, acetogens; blue, methanogens. *Methanosaetaceae* are in quotes due to its current status as an illegitimate name (http://www.bacterio.net).

Anaerovorax, *Christensenellaceae*, *Lachnospiraceae*, and *Peptostreptococcaceae* were identified as fermenters in gut contents but were not detected or not associated with fermenters in mire 'soils' (Figure 62, Figure 66). Acetogenesis and methanogenesis have been thought to be unimportant in the earthworm gut (Hornor and Mitchell 1981, Karsten and Drake 1997) but have been observed to be stimulated by H_2-CO_2 and formate in gut contents of the earthworm *E. eugeniae* (Figure 50, Figure 51). Acetogens related to *Terrisporobacter*, *Blautia*, and *Marvinbryantia* and methanogens related to *Methanobacteriaceae*, *Methanomicrobiaceae*, *"Methanosaetaceae"*, *Methanosarcinaceae*, and one family-level phylotype have been identified in gut contents (Figure 66), indicating that the impact of acetogenesis and methanogenesis might depend on the species, source of food, and/or feeding preference of the earthworm.

With lower termites, it has been observed that collectively gut-derived microorganisms degrade complex organic matter and mainly acetogenesis-derived acetate is absorbed by the termite (Brune 2014). Together with acetate also other short-chain fatty acids are absorbed by termites (Brune 2014). Succinate, propionate, and butyrate were produced from supplemental glucose in gut contents of the earthworm *E. eugeniae* (Figure 51). Thus, potentially fermentation-derived succinate, propionate, and butyrate in the earthworm gut (Figure 66) might be absorbed by the earthworm and similar interactions such as observed with lower termites between acetogens, other microorganisms and the termite might exist with certain earthworm species and could be addressed in future studies.

Formate has been identified as an important intermediate in mire 'soil' and especially in the root zone of mire plants (Figure 65). In gut contents, formate was detected as a glucose-derived fermentation product, and formate-utilizing acetogens and methanogens were likewise detected, indicating that formate could be an important intermediate in gut contents of the earthworm *E. eugeniae*. Even so, the role of formate as an intermediate forming a trophic link between FHL-containing fermenters, acetogens and methanogens for the earthworm gut has

not been investigated in this dissertation, future studies could highlight the importance of formate in this methanogenic food web.

Acetogens are commonly thought of as being O_2-sensitive strict anaerobes, and the association of acetogens with wetland plant roots and aerated forest soil might therefore be considered a paradox since roots periodically leak O_2 (Armstrong *et al.* 1991, Kraemer and Alberte 1995). However, acetogens isolated from roots and other habitats subjected to aeration have various mechanisms for dealing with oxidative stress, such as forming commensal partnerships with aerotolerant fermentative microorganisms that can consume O_2 and thereby protect the acetogen from oxidative stress (Figure 61, Gößner *et al.* 1999, Gößner *et al.* 2006, Küsel *et al.* 2001). In these commensal interactions, the fermentative partner also forms products such as H_2, formate, lactate, and ethanol that can be subsequently utilized by the acetogen. The current study indicates that acetogens can utilize formate-derived H_2 that is produced by FHL-containing fermenters and fermentation-derived compounds. Those observations provide further insights into the interactions of acetogens with other functional groups of microorganisms.

Stable isotope probing was used to identify active microorganisms in mire 'soil' and gut contents of *E. eugeniae* (5.3, 5.5.3). This method is based on the assumption that microorganism dissimilate a certain compound (e.g., ferment glucose) and also assimilate that compound (e.g., synthesis of DNA and RNA). In this dissertation, those compounds were either supplemental [^{13}C]formate and [^{13}C]glucose or ^{13}C-enriched dissimilation products derived from [^{13}C]formate and [^{13}C]glucose consumption (i.e., cross-feeding, Neufeld *et al.* 2007a). The attempt to label acetogens with [^{13}C]formate in mire 'soil' was not successful, likely due to (a) dissimilation and assimilation of [^{13}C]formate being uncoupled (e.g., formate might have been dissimilated but an endogenous, non-labeled carbon source such as glucose was assimilated), (b) labeling might not have been sufficient, or (c) 'soil' samples for molecular

analysis might not have contained labeled acetogens (e.g., gene analysis of roots yielded 16S rRNA gene sequences closely related to known acetogens, indicating that roots contain acetogens but the sampling of 'soil' slurries with syringe and needle would collect predominantly soil particles but not roots). Many acetogens are non-monophyletic, i.e., are phylogenetically distributed with non-acetogens in the same genera (Drake *et al.* 2008, Drake and Küsel 2005, Drake *et al.* 2006), a factor complicating their assessment by standard *fhs* and 16S rRNA gene analysis. Several organisms originally described as non-acetogens have later been discovered to be acetogenic (e.g., *T. glycolicus* [Drake *et al.* 2006, Drake and Küsel 2005, Küsel *et al.* 2001]), and thus, raising the question as to whether any of the detected non-acetogenic taxa might contain heretofore unknown acetogenic capabilities. A recent study in which new *fhs* primers were developed for accessing acetogens in the rumen identified potential acetogens that were not closely related to known acetogens (Henderson *et al.* 2010). Novel acetogens such as *Alkalibaculum bacchi* (Allen *et al.* 2010), *Moorella perchloratireducens* (Balk *et al.* 2008), and isolate TWA4 belonging to *Lachnospiraceae* (Gagen *et al.* 2014) have been isolated recently. Those findings illustrate (a) the difficulties to identify acetogens with molecular methods and (b) the existence of hitherto unknown acetogens in various ecosystems which reinforces the likelihood that hitherto unknown acetogens contribute to the formation of acetate in mire 'soils', the mire rhizosphere, and the earthworm gut.

The collective results indicated that microbial processes driving methanogenesis in mire 'soils', rhizosphere of mire plants, and gut contents of *E. eugeniae* are qualitatively more similar than dissimilar but are facilitated by dissimilar microbial communities. The functional redundancy (Miki *et al.* 2014) of the microbial communities is particularly reflected in the large number of the detected fermentative taxa that are not identical in each of the anoxic habitats but nonetheless catalyze similar processes (Figure 62, Figure 66, 6.1.2). Functional redundancy of microorganisms in other habitats has been observed, for example, for diverse

members of *Rhodocyclales* in activated sludge (Hesselsoe *et al.* 2009) or for the bacterial and archaeal community in mined, restored, and natural peatlands (Basiliko *et al.* 2013). A minority of nine family-level phylotypes that harbor potential fermenters were shared between contrasting mire 'soils' and mire-derived roots (i.e., *Acidobacteriaceae*, *Chitinophagaceae*, *Clostridiaceae*, *Holophagaceae*, *Neisseriaceae*, *Opitutaceae*, *Peptococcaceae*, *Ruminococcaceae*, and *Veillonellaceae*), indicating that those taxa contribute to fermentation on roots and surrounding soils. Some family-level 16S rRNA gene phylotypes that were affiliated with taxa that are capable of fermentation were detected with mire-derived roots but were not detected in 'soils' of analyzed contrasting mires (5.2), such as *Bacteroidaceae*, *Campylobacteraceae*, *Enterobacteriaceae*, *Lachnospiraceae*, *Marinilabiliaceae*, *Porphyromonadaceae*, *Roseiarcaceae*, indicating that roots of mire plants harbor different microbial communities than the surrounding soil. In general mire 'soil' and roots of wetland plants shared more common phylotypes than with gut contents of the earthworm *E. eugeniae* (Figure 67). A minority of phylotypes were detected in all three habitats: *Acetobacteraceae*, *Bradyrhizobiaceae*, *Clostridiaceae*, *Methanobacteriaceae*, "*Methanosaetaceae*", *Methanosarcinaceae*, *Rhodospirillaceae*, *Ruminococcaceae*, and *Solirubrobacteraceae*. Those taxa are capable of N_2-fixation, fermentation, aerobic respiration, anaerobic respiration and methanogenesis.

Methanogens that can grow with H_2-CO_2 were detected in similar relative abundance and accounted for 56-87 % in mire 'soils', 74 % with *Carex* roots, and 96-99 % in gut contents of *E. eugeniae* (6.5). Many of the detected family-level *mcrA* phylotypes were detected in all three habitats but on a species level only *Methanosaeta concilii* was detected in mire 'soil', *Carex* roots, and gut contents of *E. eugeniae* (Figure 24, Figure 40, Figure 46). *Methanolinea mesophila* and *Methanosarcina vacuolata* were only detected in mire 'soils' (Figure 24). *Methanosarcina horonobensis* and *Methanobacterium lacus* were only detected with *Carex* roots (Figure 40). Species of *Methanoculleus*, *Methanosarcina mazei*, and *Methanobacterium palustre* were only detected in gut contents of *E. eugeniae* (Figure 46). These findings illustrate the functional redundancy of methanogens in contrasting CH_4-emitting habitats.

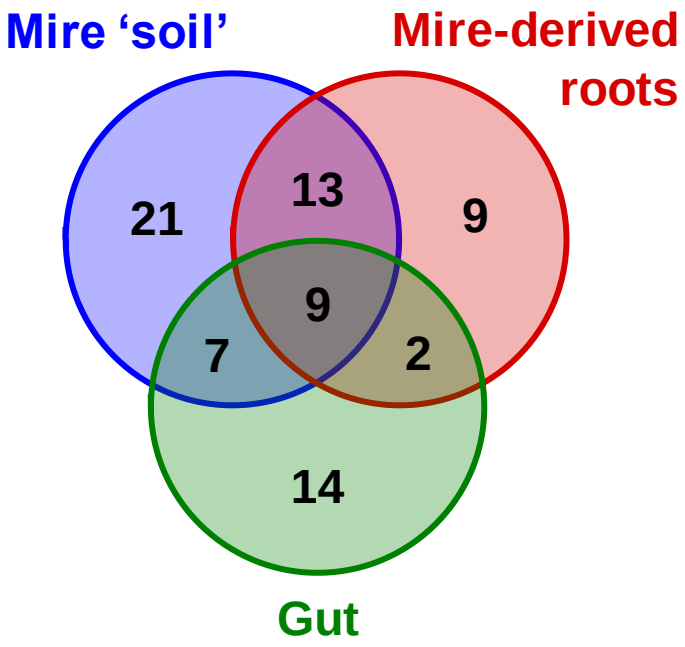


Figure 67: Venn diagram of archaeal and bacterial family-level phylotypes that were detected in mire 'soil', mire-derived roots, and gut contents of *E. eugeniae*.

Diagram includes total number of detected phylotypes that were affiliated with classified taxa from (a) mire 'soils' and 'soil' slurries with and without supplementations (Figure 24, Table 31), (b) *Carex* roots and a mixture of mire-derived roots and root slurries with and without supplementations (Figure 39, Table 36), and (c) gut contents and slurries with gut contents from *E. eugeniae* with and without supplementation (Figure 48, Table 44).

The analyzed processes are parts of complex trophically linked food webs. Those processes and associated taxa were assessed under laboratory conditions and thus might not reflect *in situ* field conditions but rather indicate potentials of anaerobic processes and microbial communities. A more extensive sequencing of the microbial communities will be required to gain a more complete understanding of how bacterial and archaeal species-level diversities differ in contrasting anoxic habitats. Likewise, transcriptomic or proteomic analyses would provide insight on which taxa respond to a particular *in situ* condition, seasonal effects, or perturbation thereof. In this regard, non-methanogenic archaea were not assessed, and although bacteria (e.g., fermenters and acetogens) are conceived to be important to the intermediary production of methanogenic substrates and thus significant drivers of methanogenesis, non-methanogenic archaea may also catalyze such intermediary processes (Zehnder 1978, McInerney and Bryant 1981, Drake *et al.* 2009). Nonetheless, within the constraints of these limitations, this dissertation gives insights into the broad metabolic

diversity of bacterial communities and the functional redundancy of anaerobes in mire ‘soils’, rhizosphere, and gut contents of *E. eugeniae*, and extended previous findings on fermentation, acetogenesis, and methanogenesis in those contrasting habitats (Drake and Horn 2007, Drake *et al.* 2009, Schulz *et al.* 2015).

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8 Publications and Presentations

Data presented in this dissertation has been published or is in preparation to be published. Published data is indicated with the corresponding reference throughout this dissertation.

8.1 Publications to date

Hunger S, O Schmidt, AS Gößner, and HL Drake (2016) Formate-derived H_2, a driver of hydrogenotrophic processes in the root-zone of a methane-emitting fen. Environ Microbiol (in press, doi: 10.1111/1462-2920.13301).

Hunger S, AS Gößner, and HL Drake (2015) Anaerobic trophic interactions of contrasting methane-emitting mire soils: processes versus taxa. FEMS Microbiol Ecol 91: fiv045.

Schulz K, S Hunger, GG Brown, SM Tsai, CC Cerri, R Conrad, and HL Drake (2015) Methanogenic food web in the gut contents of methane-emitting earthworm *Eudrilus eugeniae* from Brazil. ISME J 9: 1778-92.

Antony CP, D Kumarsan, S Hunger, HL Drake, JC Murrell, and YS Shouche (2012) Microbiology of Lonar Lake and other soda lakes. ISME J 7: 468-76.

Depkat-Jakob PS, S Hunger, K Schulz, GG Brown, SM Tsai, and HL Drake (2012) Emission of methane by *Eudrilus eugeniae* and other earthworms from Brazil. Appl Environ Microbiol 78: 3014-9.

Hunger S, AS Gößner, and HL Drake (2011) Trophic links between the acetogen *Clostridium glycolicum* KHa and the fermentative anaerobe *Bacteroides xylanolyticus* KHb, isolated from Hawaiian forest soil. Appl Environ Microbiol 77: 6281-5.

Hunger S, O Schmidt, M Hilgarth, MA Horn, S Kolb, R Conrad, and HL Drake (2011) Competing formate- and carbon dioxide-utilizing prokaryotes in an anoxic methane-emitting fen soil. Appl Environ Microbiol 77: 3773-85.

8.2 Manuscripts in preparation

Hunger S, AS Gößner, C Bruß, and HL Drake (2016) Acetogenesis and other anaerobic processes in the gut of the methane-emitting earthworm *Eudrilus eugeniae*. ISME J (*in preparation*).

Meier A, S Hunger, and HL Drake (2016) Differential engagement of diverse sugar driven fermentations and associated communities in the earthworm gut. Appl Environ Microbiol (*in preparation*).

8.3 Invited talks

Hunger S, O Schmidt, M Hilgarth, AS Gößner, and HL Drake (2014) Potential interactions of anaerobes in fen rhizospheres. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. DEV05, p 88.

Hunger S (2013) Thermodynamics and Gibbs free energy. Chinese Academy of Sciences, Beijing, China.

Hunger S (2013) The application of stable isotope probing in a methane-emitting fen soil. Chinese Academy of Sciences, Beijing, China.

Hunger S, C Burger, C Emmerich, M Eppendorfer, AS Gößner, and HL Drake (2012) Abundance and diversity of methanogens in German fen and peat bog soils. Abstract for the 'BayCEER Workshops', Abstr. O2.4, www.bayceer.uni-bayreuth.de/ws2012/. *with presentation award*

Hunger S, O Schmidt, M Hilgarth, MA Horn, S Kolb, and HL Drake (2011) Anaerobic formate- and CO_2-assimilating prokaryotic taxa in an anoxic methane-emitting fen soil. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. EMV001, p 82.

8.4 Poster presentations

Drake HL, S Hunger, O Schmidt, and AS Gößner (2016) Formate, a driver of anaerobic processes in the rhizosphere of a methane-emitting fen. Abstract for the conference 'ASM Microbe 2016', Abstr. 569.

Hunger S, C Bruß, R Conrad, and HL Drake (2016) Acetogenesis and fermentation in the gut of the methane-emitting earthworm *Eudrilus eugeniae*. Abstract for the conference the 'ASM Microbe 2016', Abstr. 613.

Hunger S, C Bruß, R Conrad, and HL Drake (2016) Acetogenesis and other anaerobic processes in the gut of the methane-emitting earthworm *Eudrilus eugeniae*. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. EMP31, p 189.

Meier A, S Hunger, and HL Drake (2016) Differential engagement of diverse sugar driven fermentations and associated communities in the earthworm gut. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. EMP36, p 191.

Hunger S, C Bruß, R Conrad, and HL Drake (2015) Acetogens in the methanogenic food web of the methane-emitting earthworm *Eudrilus eugeniae*. Abstract for the 'Gordon Research Conference – Applied and Environmental Microbiology'.

Hunger S, K Schulz, AS Gößner, GG Brown, SM Tsai, CC Cerri, and HL Drake (2014) Anaerobic processes in gut contents of the methane-emitting earthworm *Eudrilus eugeniae*. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. HMP58, p 270.

Hunger S, O Schmidt, M Hilgarth, AS Gößner, and HL Drake (2014) Hydrogenase-containing taxa, acetogens, and methanogens are linked in the rhizosphere of fen-derived

plants. Abstracts for the 'Gordon Research Conference – Molecular Basis of Microbial One-Carbon Metabolism'.

Schulz K, S Hunger, GG Brown, SM Tsai, and HL Drake (2014) Methanogenesis and associated prokaryotes in gut contents of the methane-emitting earthworm *Eudrilus eugeniae* from Brazil. Abstract for the '114th General Meeting of the American Society of Microbiology 2014', Abstr. 1303, p 158.

Hunger S, C Burger, AS Gößner, and HL Drake (2014) Intermediary ecosystem metabolism and methanogenesis of contrasting wetland soils. Abstract for the '114th General Meeting of the American Society of Microbiology 2014', Abstr. 1791, p 178.

Hunger S, C Burger, AS Gößner, and HL Drake (2013) Differential trophic interactions and microbial communities of contrasting methane-emitting peatland soils. Abstract for the 'Gordon Research Conference – Applied and Environmental Microbiology'.

Hunger S, AS Gößner, and HL Drake (2012) Acetogens and formate-hydrogen-lyase-containing taxa are linked in the rhizosphere of fen-derived plants. Abstract for the 'Gordon Research Conference - Molecular Basis of Microbial One-Carbon Metabolism'.

Hunger S, M Eppendorfer, C Bruß, AS Gößner, and HL Drake (2012) Intermediary ecosystem metabolism of methane-emitting fen and peat bog soils. Abstract for the '112th General Meeting of the American Society of Microbiology 2012', Abstr. 334, p 112.

Hunger S, C Bruß, M Eppendorfer, AS Gößner, and HL Drake (2012) Intermediary ecosystem metabolism in different CH_4-emitting peatland soils. Abstract for the conference of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie', Abstr. SMP002, p 214.

Hunger S, AS Gößner, and HL Drake (2011) Trophic interactions between the acetogen *Clostridium glycolicum* KHa and the fermentative anaerobe *Bacteroides xylanolyticus* KHb isolated from Hawaiian forest soil: a xylan-utilizing partnership. Abstract for the international symposium 'Extremophiles: Key to Bioenergy?', p 44.

Hunger S, O Schmidt, M Hilgarth, MA Horn, S Kolb, and HL Drake (2011) Formate- and carbon dioxide-utilizing bacteria and archaea in methane-emitting peatland soil. Abstract for the '1st International Conference on Biogas Microbiology', Abstr. P-22, p 87. *with poster award*

Hunger S, AS Gößner, and HL Drake (2011) Trophic interaction between the acetogen *Clostridium glycolicum* KHa and the aerotolerant fermentative anaerobe *Bacteroides xylanolyticus* KHb isolated from Hawaiian forest soil. Abstract for the 'Gordon Research Conference - Molecular Basis of Microbial One-Carbon Metabolism'.

Hunger S, O Schmidt, M Hilgarth, MA Horn, S Kolb, and HL Drake (2011) Formate- and carbon dioxide-utilizing prokaryotes in an anoxic methane-emitting fen soil. Abstract for the '111th General Meeting of the American Society of Microbiology 2011', Abstr. N-310.

9 Acknowledgements

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10 Appendix

Table 46: Sequences used for similarity plots of *fhs* and corresponding 16S rRNA gene sequences.[a]

	Accession numbers	
Taxa	*fhs*	16S rRNA gene
Acetobacterium carbinolicum	DQ152908	X96956
Acetobacterium psammolithicum	AJ494824	AF132739
Acetobacterium woodii	AF295701	X96954
Acholeplasma laidlawii	NC_010163	CP000896
Acidiphilium cryptum	CP000697	CP000697
Acidobacterium capsulatum	NC_012483	CP001472
Actinobacillus pleuropneumoniae	NC_9053	CP000569
Actinomyces urogenitalis	NZ_ACFH01000006	ACFH01000038
Aeromonas hydrophila	NC_8570	AY987754
Aeromonas salmonicida	NC_9348	CP000644
Agrobacterium radiobacter	CP000628	CP000628
Akkermansia muciniphila	CP001071	CP001071
Aliivibrio salmonicida	NC_11312	FM178379
Alistipes putredinis	NZ_ABFK02000017	ABFK02000016
Alkaliphilus metalliredigens	CP000724	CP000724
Anaerocellum thermophilum	NC_12034	CP001393
Anaerococcus lactolyticus	NZ_ABYO01000281	ABYO01000217
Anaerococcus tetradius	NZ_ACGC01000008	ACGC01000107
Anaerostipes caccae	NZ_ABAX03000038	ABAX03000023
Anaerotruncus colihominis	ABGD02000027	ABGD02000021
Arthrobacter aurescens	NC_8711	CP000474
Arthrobacter chlorophenolicus	CP001341	AF102267
Arthrobacter sp. FB24	CP000454	CP000454
Atopobium rimae	NZ_ACFE01000001	ACFE01000007
Atopobium vaginae	NZ_ACGK01000045	ACGK01000003
Bacillus anthracis	NC_12581	CP001215
Bacillus cereus	NC_6274	CP000001
Bacillus coagulans	NZ_AAWV01000108	AAWV02000001
Bacillus pumilus	NC_9848	AY167879
Bacillus thuringiensis	NC_5957	AB116122
Bacillus weihenstephanensis	CP000903	CP000903

Taxa	Accession numbers	
	fhs	16S rRNA gene
Bacteroides cellulosilyticus	NZ_ACCH01000370	ACCH01000108
Bacteroides fragilis	NC_3228	CR626927
Bacteroides pectinophilus	NZ_ABVQ01000035	ABVQ01000036
Bacteroides stercoris	NZ_ABFZ02000018	ABFZ02000010
Bartonella tribocorum	AM260525	AM260525
Beijerinckia indica	CP001016	CP001016
Bifidobacterium adolescentis	AP009256	AP009256
Bifidobacterium animalis	NC_11835	CP001213
Bifidobacterium breve	NZ_ACCG01000036	ACCG02000012
Bifidobacterium catenulatum	NZ_ABXY01000023	ABXY01000019
Bifidobacterium dentium	NZ_ABIX02000002	ABIX02000002
Bifidobacterium gallicum	NZ_ABXB01000003	ABXB03000004
Bifidobacterium pseudocatenulatum	NZ_ABXX02000001	ABXX02000002
Blautia hydrogenotrophica	NZ_ACBZ00000000	ACBZ01000217
Blautia producta	AF295707	AB196512
Caldanaerobacter subterraneus	AE008691	AE008691
Campylobacter concisus	CP000792	CP000792
Campylobacter curvus	NC_9715	CP000767
Campylobacter rectus	NZ_ACFU01000002	ACFU01000050
"*Candidatus* Pelagibacter sp. HTCC7211"	DS995298	ABVS01000001
Capnocytophaga sputigena	NZ_ABZV01000007	ABZV01000054
Carboxydibrachium pacificum	NW_2243368	ABXP01000185
Carboxydothermus hydrogenoformans	CP000141	CP000141
Chloroflexus aggregans	CP001337	CP001337
Chloroflexus aurantiacus	NC_10175	CP000909
Chloroflexus sp. Y400fl	NC_12032	CP001364
Clostridium aceticum	AF295705	Y18183
Clostridium asparagiforme	ACCJ01000481	ACCJ01000522
Clostridium beijerinckii	CP000721	CP000721
Clostridium botulinum	NZ_ABDQ01000008	ABDQ01000004
Clostridium carboxidivorans	ZP_5391913	ACVI01000229
Clostridium cellulolyticum	NC_11898	CP001348
Clostridium difficile	NC_9089	AM180355
Clostridium formicaceticum	AF295702	X77836
Clostridium glycolicum CA6	GU124152	X76750

Taxa	Accession numbers	
	fhs	16S rRNA gene
Clostridium hylemonae	NZ_ABYI02000023	AB117569
Clostridium kluyveri	NC_9706	CP000673
Clostridium magnum	AF295703	GU129927
Clostridium methylpentosum	NZ_ACEC01000021	ACEC01000059
Clostridium novyi	NC_8593	CP000382
Clostridium perfringens	NC_8261	ABDV01000010
Clostridium phytofermentans	NC_10001	CP000885
Clostridium scindens	NZ_ABFY02000009	ABFY02000057
Clostridium sp. M62/1	NZ_ACFX01000085	ACFX01000080
Clostridium sp. SS2/1	NZ_ABGC03000031	ABGC03000041
Clostridium thermocellum	NC_9012	CP000568
Coprothermobacter proteolyticus	NC_11295	CP001145
Corynebacterium aurimucosum	NC_12590	AJ309207
Corynebacterium diphtheriae	NC_2935	BX248356
Corynebacterium jeikeium	CR931997	CR931997
Corynebacterium matruchotii	NZ_ACEB01000021	ACEB01000045
Desulfatibacillum alkenivorans	NC_11768	CP001322
Desulfitobacterium hafniense	NC_11830	AF403181
Desulfobacterium autotrophicum	CP001087	CP001087
Desulfococcus oleovorans	CP000859	CP000859
Desulfomicrobium baculatum	AJ494755	AF030438
Desulfonatronospira thiodismutans	NZ_ACJN01000001	ACJN01000005
Desulfotomaculum reducens	CP000612	CP000612
Desulfovibrio desulfuricans	CP001358	CP001358
Desulfovibrio gigas	AJ494759	DQ447183
Desulfovibrio piger	AJ494750	AF192152
Desulfovibrio salexigens	AJ494751	CP001649
Desulfovibrio vulgaris	AJ494752	DQ826728
Dethiobacter alkaliphilus	NZ_ACJM01000014	ACJM01000032
Dictyoglomus thermophilum	NC_11297	CP001146
Dictyoglomus turgidum	CP001251	CP001251
Dinoroseobacter shibae	CP000830	CP000830
Dorea formicigenerans	NZ_AAXA02000014	AAXA02000006
Elusimicrobium minutum	NC_10644	CP001055
Enterococcus faecalis	NZ_ACIX01000064	ACIX01000001

Taxa	Accession numbers	
	fhs	16S rRNA gene
Enterococcus faecium	NZ_ACHL01000094	ACHL01000045
Eubacterium acidaminophilum	AY722711	AF071416
Eubacterium biforme	NZ_ABYT01000111	ABYT01000002
Eubacterium hallii	NZ_ACEP01000047	ACEP01000116
Eubacterium limosum	AF295706	AB298909
Exiguobacterium sp. AT1b	NZ_ABPF01000008	CP001615
Finegoldia magna	ACHM01000230	ACHM01000169
Fusobacterium nucleatum	CM000440	AABF01000001
Gardnerella vaginalis	NZ_ACGF01000006	ACGF01000131
Gemmatimonas aurantiaca	AP009153	AP009153
Granulibacter bethesdensis	YP_743868	CP000394
Haemophilus ducreyi	NC_2940	AE017143
Heliobacterium modesticaldum	NC_10337	CP000930
Herpetosiphon aurantiacus	CP000875	CP000875
Hyphomicrobium denitrificans	ZP_5376333	ACVL01000012
Hyphomonas neptunium	NC_8358	CP000158
Labrenzia aggregata	ZP_1545457	AAUW01000002
Labrenzia alexandrii	ZP_5116914	ACCU01000015
Lactobacillus brevis	NZ_ACGG01000118	ACGG01000095
Lactobacillus buchneri	NZ_ACGH01000132	ACGH01000101
Lactobacillus casei	FM177140	FM177140
Lactobacillus delbrueckii	NC_8054	AB008207
Lactobacillus fermentum	NZ_ACGI01000041	ACGI01000130
Lactobacillus gasseri	NZ_ACGO01000006	ACGO01000023
Lactobacillus hilgardii	NZ_ACGP01000185	ACGP01000200
Lactobacillus johnsonii	ACGR01000038	ACGR01000047
Lactobacillus paracasei	NZ_ABQV01000011	ABQV01000067
Lactobacillus plantarum	ACGZ01000008	ACGZ01000098
Lactobacillus reuteri	AAPZ02000001	AAPZ02000001
Lactobacillus ruminis	NZ_ACGS01000013	ACGS01000025
Lactobacillus sakei	NC_7576	CR936503
Lactobacillus salivarius	ACGT01000002	AF089108
Lactobacillus vaginalis	NZ_ACGV01000115	ACGV01000168
Lactococcus lactis	NC_2662	AE005176
Laribacter hongkongensis	NC_12559	CP001154

Taxa	Accession numbers	
	fhs	16S rRNA gene
Leuconostoc citreum	DQ489736	DQ489736
Leuconostoc mesenteroides	NZ_ACKV01000069	CP000414
Listeria monocytogenes	NC_2973	AE017262
Listeria welshimeri	NC_8555	AM263198
Maricaulis maris	YP_756642	CP000449
Marinitoga piezophila	NW_2435198	ABXR01000030
Mesorhizobium loti	NP_104026	BA000012
Mesorhizobium opportunistum	ZP_5810756	ACZA01000068
Methylibium petroleiphilum	YP_1022452	AF176594
Methylobacillus flagellatus	CP000284	CP000284
Methylobacterium chloromethanicum	NC_11757	AF198624
Methylobacterium extorquens	CP000908	CP000908
Methylobacterium nodulans	NC_11894	CP001349
Methylobacterium populi	CP001029	CP001029
Methylobacterium radiotolerans	CP001001	CP001001
Methylobacterium sp. 446	CP000943	CP000943
Methylococcus capsulatus	NC_2977	AE017282
Methylophaga thiooxidans	NW_2475443	ABXT01000015
Mitsuokella multacida	NZ_ABWK01000014	ABWK02000005
Moorella thermoacetica	NC_7644	CP000232
Myxococcus xanthus	NC_8095	CP000113
Natranaerobius thermophilus	NC_10718	CP001034
Neisseria flavescens	NZ_ACEN01000025	ACEN01000027
Neisseria lactamica	NZ_ACEQ01000038	AJ239283
Neisseria meningitidis	AL157959	AL157959
Nocardioides sp. JS614	NC_8699	CP000509
Opitutus terrae	CP001032	CP001032
Oribacterium sinus	ACKX01000049	ACKX01000142
Oxobacter pfennigii	KPU46025	X77838
Parabacteroides johnsonii	NZ_ABYH01000144	ABYH01000014
Paracoccus denitrificans	CP000489	CP000489
Petrotoga mobilis	CP000879	CP000879
Photobacterium profundum	CR378672	AB003191
Porphyromonas gingivalis	NC_2950	AB035456
Prevotella copri	NZ_ACBX01000107	ACBX02000014

Taxa	Accession numbers	
	fhs	16S rRNA gene
Propionibacterium acnes	NZ_ABZW01000003	ABZW01000012
Proteus mirabilis	NZ_ACLE01000036	ACLE01000013
Proteus vulgaris	AF295710	DQ885257
Pseudovibrio sp. JE062	DS996805	ABXL01000006
Psychrobacter sp. PRwf1	CP000713	CP000713
Rhizobium etli	NC_10994	CP001074
Rhizobium leguminosarum	AM236080	AM236080
Rhizobium sp. NGR234	CP001389	AY260147
Rhodobacter sphaeroides	CP000661	CP000144
Roseiflexus castenholzii	CP000804	CP000804
Roseiflexus sp. RS1	CP000686	CP000686
Roseobacter denitrificans	NC_8209	CP000362
Roseovarius nubinhibens	ZP_958814	AALY01000001
Rubrobacter xylanophilus	CP000386	CP000386
Ruegeria sp. R11	DS999054	ABXM01000001
Ruminococcus lactaris	NZ_ABOU02000048	ABOU02000049
Ruminococcus torques	NZ_AAVP02000021	AAVP02000002
Saccharopolyspora erythraea	NC_9142	AM420293
Shewanella baltica	CP000563	CP000563
Shewanella halifaxensis	CP000931	CP000931
Shewanella loihica	CP000606	CP000606
Shewanella oneidensis	NC_4347	AE014299
Shewanella pealeana	CP000851	CP000851
Shewanella putrefaciens	CP000681	CP000681
Shewanella sp. W3181	CP000503	CP000503
Shewanella woodyi	CP000961	CP000961
Sinorhizobium medicae	CP000738	CP000738
Sinorhizobium meliloti	AL591688	AL591688
Spiroplasma citri	AM285305	AM285316
Sporomusa ovata	AF295708	AJ279800
Sporomusa termitida	AF295709	M61920
Staphylococcus aureus	NC_2952	BX571856
Staphylococcus capitis	ACFR01000005	ACFR01000029
Staphylococcus carnosus	NC_12121	AM295250
Staphylococcus epidermidis	NC_2976	CP000029

Taxa	Accession numbers	
	fhs	16S rRNA gene
Staphylococcus haemolyticus	NC_7168	AP006716
Streptococcus agalactiae	NC_7432	CP000114
Streptococcus equi	NC_12470	FM204884
Streptococcus infantarius	NZ_ABJK02000020	ABJK02000007
Streptococcus mutans	AE014133	AE014133
Streptococcus pneumoniae	NC_3098	AF003930
Streptococcus pyogenes	AE004092	AB002521
Streptococcus sanguinis	CP000387	CP000387
Streptococcus suis	NZ_AAFA03000003	AAFA03000005
Streptococcus thermophilus	NC_6449	CP000024
Syntrophobacter fumaroxidans	CP000478	CP000478
Thermoanaerobacter kivui	AF295704	L09160
Thermoanaerobacter pseudethanolicus	NC_10321	CP000924
Thermoanaerobacter sp. X514	NC_10320	CP000923
Thermodesulfovibrio yellowstonii	NC_11296	CP001147
Thermomicrobium roseum	NC_11959	CP001275
Thermosinus carboxydivorans	NZ_AAWL01000002	AAWL01000001
Thermotoga sp. RQ2	CP000969	AJ872273
Thermus aquaticus	NZ_ABVK02000006	ABVK02000001
Treponema denticola	NC_2967	AE017226
Treponema primitia	AJ494823	AF093251
Ureaplasma parvum	NC_10503	AF073456
Ureaplasma urealyticum	NZ_AAYN02000002	AAYN02000002
Vibrio cholerae	NC_12580	CP001233
Vibrio fischeri	NC_6840	CP000020
Vibrio harveyi	NZ_AAWP01000011	AAWP01000045
Vibrio parahaemolyticus	NW_2475411	ACCV01000071
Vibrio sp. Ex25	DS267821	AAKK02000060
Xanthobacter autotrophicus	CP000781	CP000781

[a] Sequences were plotted in Figure 17.

Table 47: Sequences used for similarity plots of *mcrA* and corresponding16S rRNA gene sequences.[a]

	Accession numbers	
Taxa	*mcrA*	16S rRNA gene
Methanobacterium beijingense	EF465106	AY552778
Methanobacterium bryantii	AF313806	AF028688
Methanobacterium formicicum DSM1535	EF465108	NR_025028
Methanobacterium formicicum DSM1312	AF414050	AY196659
Methanobacterium ivanovii	EF465107	AF095261
Methanobacterium sp. HD1	AB288269	AB288265
Methanobacterium sp. MB4	DQ677519	DQ677518
Methanobacterium sp. T01	AB288286	AB288275
Methanobacterium thermaggregans	AY289750	AF095264
Methanobrevibacter arboriphilus	AF414035	AB065294
Methanobrevibacter gottschalkii	EU919431	U55239
Methanobrevibacter millerae	EU919430	AY196673
Methanobrevibacter ruminantium	AF414046	AY196666
Methanobrevibacter smithii	CP000678	CP000678
Methanobrevibacter sp. WBY1	EU919429	EU919428
Methanobrevibacter woesei	EU919432	U55237
Methanocaldococcus infernus	AY354032	AF025822
Methanocaldococcus jannaschii	NC_000909	NC_000909
Methanocella paludicola	AB300467	AB196288
Methanococcoides alaskense	AB353221	AY941802
Methanococcus aeolicus	AY354034	U39016
Methanococcus maripaludis	NC_005791	NC_005791
Methanococcus vannielii	M16893	M36507
Methanococcus voltae	X07793	U38461
Methanocorpusculum bavaricum	AF414049	AF095266
Methanocorpusculum labreanum	AY260441	AY260436
Methanocorpusculum parvum	AY260445	M59147
Methanocorpusculum sp. MSP	AY260448	AY260434
Methanocorpusculum sp. T07	AB288289	AB288279
Methanocorpusculum sp. T08	AB288290	AB288280
Methanoculleus bourgensis DSM6216	AB300786	AB065298
Methanoculleus bourgensis DSM3045	AB300787	AF095269
Methanoculleus chikugoensis	AB300779	AB038795

Taxa	Accession numbers	
	mcrA	16S rRNA gene
Methanoculleus palmolei	AB300784	Y16382
Methanoculleus sp. HC1	AB288267	AB288263
Methanoculleus sp. M07	AB288284	AB288273
Methanoculleus sp. M11	AB288285	AB288274
Methanoculleus sp. T02	AB288287	AB288276
Methanoculleus sp. T05	AB288288	AB288278
Methanoculleus sp. T14	AB288291	AB288282
Methanoculleus submarinus	DQ229156	AF531178
Methanoculleus thermophilus	AB300783	AB065297
Methanofollis liminatans	AF414041	Y16428
Methanogenium boonei	DQ229161	DQ177343
Methanogenium marinum	DQ229159	DQ177344
Methanogenium organophilum	AB353222	M59131
Methanohalophilus mahii	AB353223	M59133
Methanolinea sp. TNR	AB496719	AB447467
Methanolinea tarda	AB300466	AB162774
Methanolobus zinderi	EU715818	EU711413
Methanomethylovorans hollandica DMS1	AY260442	AF120163
Methanomethylovorans hollandica ZB	AY260437	AY260433
Methanomethylovorans thermophila	AY672820	AY672821
Methanomicrobium mobile	AF414044	AY196679
Methanopyrus kandleri	AE009439	AE009439
Methanosaeta concilii	AF414037	M59146
Methanosaeta harundinacea 8Ac	AY970348	AY817738
Methanosaeta harundinacea 6Ac	AY970349	AY970347
Methanosalsum zhilinae	AB353224	FJ224366
Methanosarcina barkeri	AY260430	CP000099
Methanosarcina lacustris FRX1	AY260443	DQ058823
Methanosarcina lacustris MM	AY260438	AY260430
Methanosarcina lacustris MS	AY260439	AY260431
Methanosarcina mazei	AB300781	AJ012095
Methanosarcina sp. HB1	AB288266	AB288262
Methanosarcina sp. HC2	AB288268	AB288264
Methanosarcina sp. T36	AB288292	AB288283
Methanosphaera stadtmanae	AF414047	AY260433

Taxa	Accession numbers	
	mcrA	16S rRNA gene
Methanosphaerula palustris	EU296536	CP001338
Methanospirillum hungatei	AF313805	CP000254
Methanothermobacter thermautotrophicus	U10036	NC_000916
Methanothermobacter thermoflexus	AY303950	X99047
Methanothermobacter thermophilus DSM6529	AY289752	X99048
Methanothermobacter wolfeii	AB300780	AB104858
Methanothermococcus okinawensis	AB353229	AB057722
Methanothermococcus thermolithotrophicus	AB353226	M59128
Methanothermus fervidus	J03375	M59145
Methanothermus sociabilis	AY289747	AF095273
Methanotorris igneus	AB353228	M59125

[a] Sequences were plotted in Figure 17.

Table 48: Class- and family-level phylotypes and relative abundances of bacterial 16S rRNA gene sequences from mire 'soils' and from slurries at the end of the 21 day incubation.

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Acidobacteria, Acidobacteria,																				
Acidobacteriaceae	-	-	-	4	-	25	14	15	20	27	29	34	39	40	43	41	32	23	24	28
Family phylotype 1[b]	-	3	-	2	6	8	-	2	-	5	7	19	4	2	13	10	2	-	-	8
Acidobacteria, Holophagae,																				
Holophagaceae	-	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-
Acidobacteria, unknown class,																				
Family phylotype 2[b]	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 3[b]	10	3	2	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 4[b]	2	5	7	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 5[b]	-	-	2	2	-	13	-	-	-	7	5	11	11	-	9	-	-	-	-	-
Family phylotype 6[b]	-	-	-	-	-	-	-	2	-	-	-	-	-	-	2	-	2	-	-	-
Actinobacteria, Actinobacteria,																				
Acidimicrobiaceae	2	10	-	6	3	-	7	2	9	2	12	11	9	10	4	2	-	-	-	5
Mycobacteriaceae	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Thermomonosporaceae	-	-	2	-	-	-	2	2	2	-	2	-	2	7	7	-	-	-	-	3
Conexibacteraceae,																				
Patulibacteraceae,	5	8	-	2	-	-	5	2	2	-	5	6	-	-	4	-	-	5	-	-
Solirubrobacteraceae[c]																				

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Actinobacteria, unknown class,																				
Family phylotype 7[b]	2	-	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 8[b]	2	3	2	4	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-
Family phylotype 9[b]	-	3	-	2	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 10[b]	2	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
Armatimonadetes, Armatimonadia,																				
Armatimonadaceae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	3
Bacteroidetes, Bacteroidia,																				
Family phylotype 11[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
Bacteroidetes, Cytophagia,																				
Cytophagaceae	-	5	2	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacteroidetes, Sphingobacteriia,																				
Chitinophagaceae	-	-	2	-	-	-	-	-	2	-	-	-	-	2	-	-	2	-	-	-
Family phylotype 12[b]	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Chlorobi, Ignavibacteria,																				
Ignavibacteriaceae	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-
Chloroflexi, Anaerolineae,																				
Anaerolineaceae	7	3	5	2	3	4	-	-	-	2	2	-	-	-	-	-	-	-	-	-
Chloroflexi, Ktedonobacteria,																				
Ktedonobacteraceae	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Family phylotype 13[b]	-	-	-	-	-	2	12	-	-	5	-	-	-	-	-	-	-	-	-	-
Family phylotype 14[b]	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroflexi, unknown class,																				
Family phylotype 15[b]	2	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 16[b]	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	3
Family phylotype 17[b]	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	3
Family phylotype 18[b]	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 19[b]	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 20[b]	-	-	-	-	-	-	-	-	2	7	-	-	-	-	-	-	-	-	-	-
Cyanobacteria, unknown class,																				
Family phylotype 21[b]	-	-	-	-	-	-	2	-	-	-	-	-	4	-	-	-	-	7	-	3
Firmicutes, Bacilli,																				
Bacillaceae	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Firmicutes, Clostridia,																				
Clostridiaceae	5	3	5	-	-	-	2	31	2	-	-	-	2	-	-	-	2	21	-	-
Peptococcaceae	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-
Ruminococcaceae	2	5	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	5	-	-
Family phylotype 22[b]	-	-	5	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Firmicutes, Negativicutes,																				
Veillonellaceae	-	-	-	-	-	-	-	2	-	7	-	-	2	-	-	-	-	7	2	10

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
"*Nitrospirae*", "*Nitrospira*", "*Nitrospiraceae*"	2	3	2	2	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
Planctobacteria, Planctomycea, *Planctomycetaceae*	2	5	-	4	-	4	10	15	20	10	5	-	11	-	-	-	7	2	-	3
Proteobacteria, Alphaproteobacteria,																				
Caulobacteraceae	-	-	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-
Beijerinckiaceae	-	-	-	2	-	2	-	-	4	2	2	4	-	-	2	2	-	2	2	-
Bradyrhizobiaceae	2	-	2	4	6	4	-	-	-	2	7	-	-	7	-	5	-	2	4	3
Hyphomicrobiaceae	2	5	15	13	6	6	5	6	2	-	-	-	2	7	4	-	-	-	-	-
Methylocystaceae	-	3	2	-	-	4	2	-	-	2	-	-	-	2	4	22	27	9	41	15
Rhizobiaceae	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xanthobacteraceae	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyloceanibacter-related phylotype	-	-	2	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetobacteraceae	-	-	-	-	3	-	-	4	-	-	-	-	-	2	-	10	7	-	22	3
Rhodospirillaceae	2	5	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-
Family phylotype 23[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Family phylotype 24[b]	-	-	2	-	3	-	-	-	-	-	2	-	-	-	-	-	2	7	2	3
Family phylotype 25[b]	-	-	-	-	-	-	-	2	7	2	7	2	4	-	-	7	-	-	2	-

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Proteobacteria, Betaproteobacteria,																				
Comamonadaceae	2	3	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxalobacteraceae	-	-	-	-	-	-	-	-	-	5	-	-	2	-	-	-	-	-	-	-
Neisseriaceae	-	-	-	-	-	-	-	2	4	-	-	-	-	-	-	-	5	5	-	-
Rhodocyclaceae	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 26[b]	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 27[b]	-	5	2	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 28[b]	5	3	5	6	21	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-
Proteobacteria, Gammaproteobacteria,																				
Coxiellaceae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Moraxellaceae	2	3	2	4	3	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 29[b]	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteobacteria, Deltaproteobacteria,																				
Bdellovibrionaceae	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
Desulfobacteraceae	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geobacteraceae	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Phaselicystidaceae, Polyangiaceae[d]	-	-	-	2	-	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-
Syntrophaceae	5	5	2	6	6	2	-	-	-	2	-	-	-	-	2	-	-	-	-	-
Syntrophobacteraceae	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Syntrophorhabdaceae	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 30[b]	-	-	-	-	-	-	-	-	2	-	-	4	-	2	2	-	-	-	-	-
Family phylotype 31[b]	2	-	2	-	3	-	2	-	2	-	-	4	-	5	-	-	-	-	-	-
Family phylotype 32[b]	2	3	-	4	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 33[b]	-	-	-	2	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spirochaetae, Spirochaetes,																				
Spirochaetaceae	2	-	-	-	-	6	-	2	2	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 34[b]	-	-	-	-	-	2	2	-	2	-	-	-	-	-	-	-	-	-	-	-
Unclassified Taxonomy,																				
Xiphinematobacter-related	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown Taxonomy[e],																				
Family phylotype 35[b]	-	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 36[b]	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 37[b]	2	-	-	2	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 38[b]	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 39[b]	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Family phylotype 40[b]	-	-	-	-	-	-	2	-	-	2	-	2	-	-	-	-	-	-	-	-
Verrucomicrobia, Opitutae,																				
Opitutaceae	-	-	-	-	-	4	-	-	2	-	-	-	7	2	-	-	-	-	-	3

Taxonomic level (phylum, class, family)	Relative abundance of 16S rRNA gene sequences (%)[a]																			
	Mire 1					Mire 2					Mire 3					Mire 4				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Verrucomicrobia, unknown class, Family phylotype 41[b]	7	3	-	2	6	6	5	4	-	2	7	2	-	2	-	-	7	-	-	3

[a] -, not detected; A, mire 'soil'; B, unsupplemented slurries; C, glucose-supplemented slurries; D, acetate-supplemented slurries; E, H_2-CO_2-supplemented slurries. Values are rounded to nearest whole number and thus might not sum up to 100 %.

[b] Sequences were considered to be a family-level phylotype without any cultured isolate if the 16S rRNA gene sequence was less than 87.5 % similar to the sequence of the closest related cultured species (Yarza *et al.* 2008).

[c] Closest related cultured species: *Conexibacter arvalis* (AB597950), 94.5-88.8 % 16S rRNA gene sequence similarity, *Patulibacter americanus* (AJ871306), 92.9-87.2 % 16S rRNA gene sequence similarity, and *Solirubrobacter soli* (AB245334), 96.8-88.1 % 16S rRNA gene sequence similarity.

[d] Closest related cultured species: *Byssovorax cruenta* (AJ833647), 88.5-86.5 % 16S rRNA gene sequence similarity, and *Phaselicystis flava* (EU545827), 90.2-87.8 % 16S rRNA gene sequence similarity.

[e] Listed family-level phylotypes do not necessarily belong to the same phylum or class.

11 (Eidesstattliche) Versicherungen und Erklärungen

(§ 5 Nr. 4 PromO)

Hiermit erkläre ich, dass keine Tatsachen vorliegen, die mich nach den gesetzlichen Bestimmungen über die Führung akademischer Grade zur Führung eines Doktorgrades unwürdig erscheinen lassen.

(§ 8 S. 2 Nr. 5 PromO)

Hiermit erkläre ich mich damit einverstanden, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung hinsichtlich der eigenständigen Anfertigung der Dissertation unterzogen werden kann.

(§ 8 S. 2 Nr. 7 PromO)

Hiermit erkläre ich eidesstattlich, dass ich die Dissertation selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

(§ 8 S. 2 Nr. 8 PromO)

Ich habe die Dissertation nicht bereits zur Erlangung eines akademischen Grades anderweitig eingereicht und habe auch nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden.

(§ 8 S. 2 Nr. 9 PromO)

Hiermit erkläre ich, dass ich keine Hilfe von gewerblichen Promotionsberatern bzw. -vermittlern in Anspruch genommen habe und auch künftig nicht nehmen werde.

…….

Ort, Datum, Unterschrift