

# Aerobic methane formation in Grey poplar plants grown under sterile conditions

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## Introduction

Objections to the experimental design of Keppler *et al.* (2006), criticizing the use of static chambers and methane-free air: e.g.,

Kirschbaum *et al.* (2006), *Functional Plant Biology* **33**: 521–530

Dueck *et al.* (2007), *New Phytologist* **175**: 29–35

No observation of aerobic methane emission from plants: e.g.,

Dueck *et al.* (2007), *New Phytologist* **175**: 29–35

Beerling *et al.* (2008), *Global Change Biology* **14**: 1821–1826

Kirschbaum & Walcroft, *Biogeosciences* **5**: 1551–1558

Observation of aerobic methane emission from plants: e.g.,

Vigano *et al.* (2008), *Biogeosciences* **5**: 937–947

Wang *et al.* (2008), *Environmental Science & Technology* **42**: 62–68

Mechanisms of aerobic methane formation: e.g.,

Keppler *et al.* (2008), *New Phytologist* **178**: 808–814

McLeod *et al.* (2008), *New Phytologist* **180**: 124–132

Messenger *et al.* (2009), *Plant, Cell & Environment* **32**: 1–9

## Open research questions

- **Missing proof for the absence of methanogenic microorganisms potentially contributing to aerobic methane emission from plants**
- **Convincing evidence that aerobic methane originates in living plant material**

## Our experimental design

- Plant species: Grey poplar (*Populus x canescens*, syn. *Populus tremula x P. alba*), derived from cell cultures under sterile conditions
- Plants on sterile medium in gas-tight flasks in CH<sub>4</sub>-free air
- Headspace was exchanged with synthetic air containing 20% of oxygen and 385 ppm <sup>13</sup>CO<sub>2</sub> (99 at% <sup>13</sup>C)
- Flasks were kept in glove box filled with pure N<sub>2</sub> for 33 days under a 16/8 h light/dark regime
- GC-IRMS analysis of methane in the headspace
- Molecular biological analysis of plant material and medium for the methyl coenzyme M reductase alpha subunit (*mcrA*) gene
- EA-IRMS of bulk plant material after end of the experiment

# Plant material



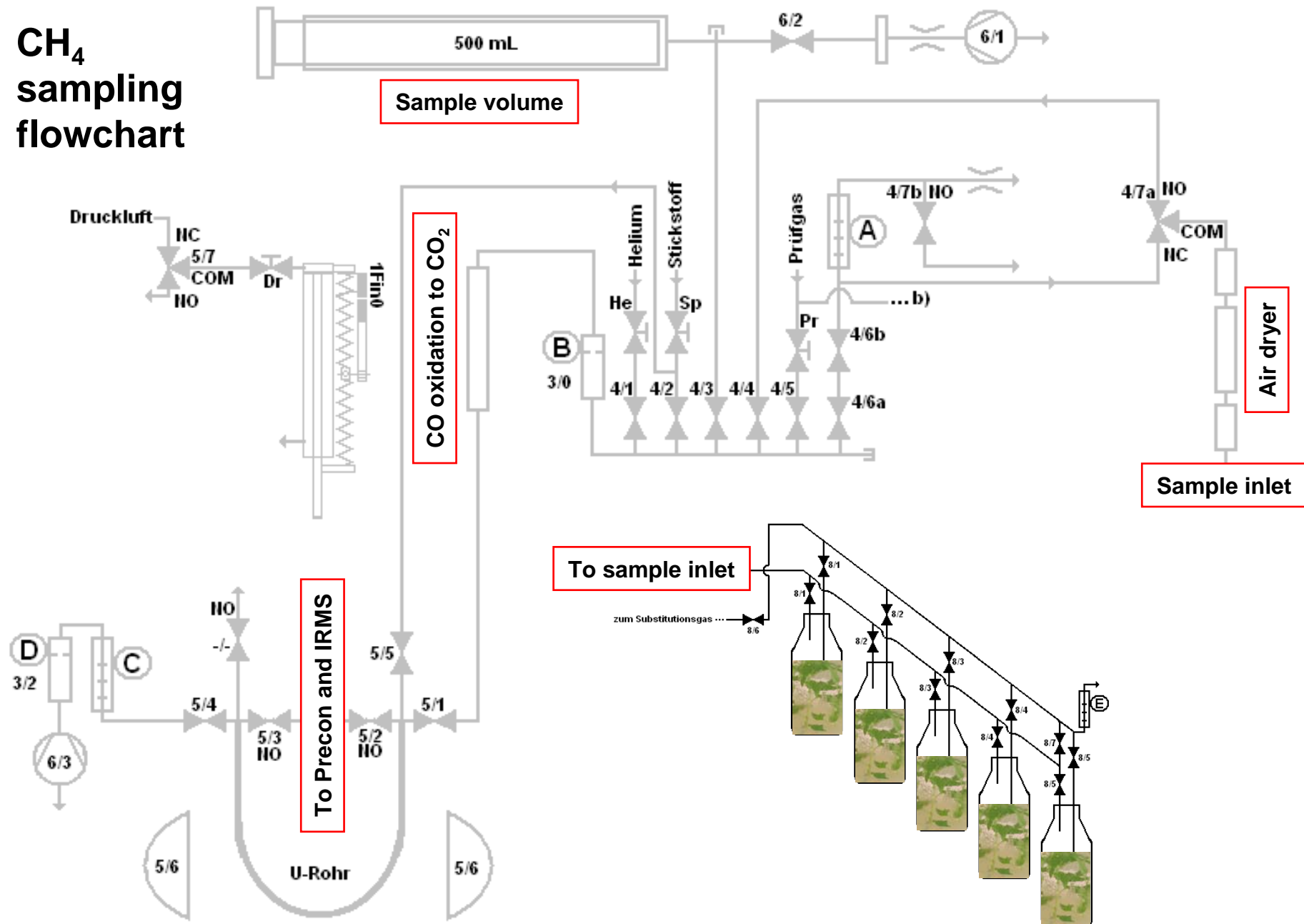
Wild type *Populus × canescens* (Aiton) Sm.  
(syn. *Populus tremula × P. alba*) lines,  
amplified by micro-propagation

7-8 plantlets were transferred under sterile  
conditions to 1-l sterile glass flasks, containing  
sterilized quartz sand and MS medium

The flasks were sealed with screw caps and  
sterilized valves; the inlet ports were  
additionally equipped with sterile filters (0.22  
 $\mu\text{m}$  pore size)

The poplar plants were grown under standard  
conditions of 27°C : 24°C (day : night) and a  
light period of 16 h with approx. 100  $\mu\text{mol m}^{-2}$   
 $\text{s}^{-1}$  photosynthetic photon flux density (PPFD)

# CH<sub>4</sub> sampling flowchart



**Condensation of sample air with freezing of CO<sub>2</sub>**

Nic  
1<sup>st</sup> workshop on aerobic methane formation, 26/27 Feb 2009, Mainz

KIT – a Cooperation between  
Karlsruhe Research Center  
and University of Karlsruhe



Forschungszentrum Karlsruhe  
in der Helmholtz-Gemeinschaft  
IMK-IFU Garmisch-Partenkirchen

# mrcA primers used for PCR

Forward primer: **GGATTCACACARTAYGGWACAGC**

981	1135
AJ584650MeThanoSphae	TAAA TAACCTTAA AA TTAT
NC_007681MeThanoSpha	TAAA TAACCTTAA AA TTAT
NC_000909MeThanoCAld	TAAA TAACCTTAA AA TTAT
NC_009637MeThanoCoCC	TAAA TAACCTTAA AA TTAT
ABF01000001MeThanoC	TAAA TAACCTTAA AA TTAT
NC_009135MeThanoCoCC	TAAA TAACCTTAA AA TTAT
NC_005791MeThanoCoCC	TAAA TAACCTTAA AA TTAT
NC_009634MeThanoCoCC	TAAA TAACCTTAA AA TTAT
ABH01000001MeThanoC	TAAA TAACCTTAA AA TTAT
NC_009635MeThanoCoCC	TAAA TAACCTTAA AA TTAT
NC_000916MeThanoTheer	TAAA TAACCTTAA AA TTAT
U09990	TAAA TAACCTTAA AA TTAT
NC_009515MeThanoSpha	TAAA TAACCTTAA AA TTAT
AY386125MeThanoACTe	TAAA TAACCTTAA AA TTAT
D0677519MeThanoACTe	TAAA TAACCTTAA AA TTAT
U10036	TAAA TAACCTTAA AA TTAT
NC_008942hypoTheTiCA	TAAA TAACCTTAA AA TTAT
NC_008942ribosomAlpr	TAAA TAACCTTAA AA TTAT
NC_007796MeThanoSpir	TAAA TAACCTTAA AA TTAT
NC_009051MeThanoCull	TAAA TAACCTTAA AA TTAT
NC_009712CandidaTusM	TAAA TAACCTTAA AA TTAT
NC_009464UnCulTuredAr	TAAA TAACCTTAA AA TTAT
AY327049unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714839unCulTuredAr	TAAA TAACCTTAA AA TTAT
NC_008553MeThanoSaeT	TAAA TAACCTTAA AA TTAT
AY260439MeThanoArC1	TAAA TAACCTTAA AA TTAT
AY260438MeThanoArC1	TAAA TAACCTTAA AA TTAT
NC_003901MeThanoArC	TAAA TAACCTTAA AA TTAT
NC_003552MeThanoArC	TAAA TAACCTTAA AA TTAT
NC_007355MeThanoArC	TAAA TAACCTTAA AA TTAT
NC_007955MeThanoCoCC	TAAA TAACCTTAA AA TTAT
NC_003551MeThanoPyru	TAAA TAACCTTAA AA TTAT
AY714816unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714825unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714837unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714852unCulTuredAr	TAAA TAACCTTAA AA TTAT
BX649197unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY327048unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714819unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714830unCulTuredAr	TAAA TAACCTTAA AA TTAT
AY714870unCulTuredAr	TAAA TAACCTTAA AA TTAT

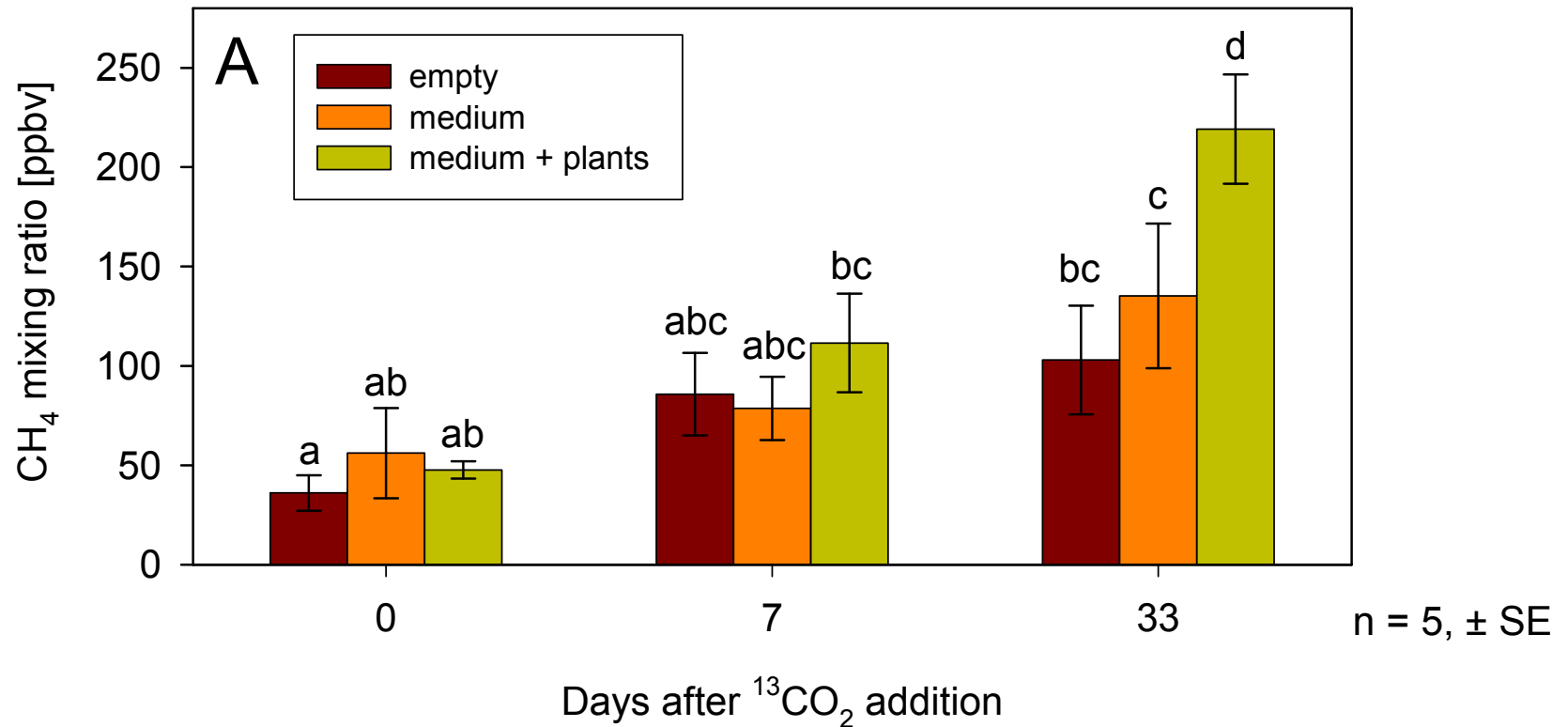
➤ Databases: thousands of mcrA sequences but only „few“ are full-length

Reverse primer: **TCATBGCRTAGTTHGGRTAGT**

1470	1624
AJ584650MeThanoSphae	CTCAGCTCTTAAA...
NC_007681MeThanoSpha	CTCAGCTCTTAAA...
NC_000909MeThanoCAld	CTCAGCTCTTAAA...
NC_009637MeThanoCoCC	CTCAGCTCTTAAA...
ABF01000001MeThanoC	CTCAGCTCTTAAA...
NC_009135MeThanoCoCC	CTCAGCTCTTAAA...
NC_005791MeThanoCoCC	CTCAGCTCTTAAA...
NC_009634MeThanoCoCC	CTCAGCTCTTAAA...
ABH01000001MeThanoC	CTCAGCTCTTAAA...
NC_009635MeThanoCoCC	CTCAGCTCTTAAA...
NC_000916MeThanoTheer	CTCAGCTCTTAAA...
U09990	CTCAGCTCTTAAA...
NC_009515MeThanoSpha	CTCAGCTCTTAAA...
AY386125MeThanoACTe	CTCAGCTCTTAAA...
D0677519MeThanoACTe	CTCAGCTCTTAAA...
U10036	CTCAGCTCTTAAA...
NC_008942hypoTheTiCA	CTCAGCTCTTAAA...
NC_008942ribosomAlpr	CTCAGCTCTTAAA...
NC_007796MeThanoSpir	CTCAGCTCTTAAA...
NC_009051MeThanoCull	CTCAGCTCTTAAA...
NC_009712CandidaTusM	CTCAGCTCTTAAA...
NC_009464UnCulTuredAr	CTCAGCTCTTAAA...
AY327049unCulTuredAr	CTCAGCTCTTAAA...
AY714839unCulTuredAr	CTCAGCTCTTAAA...
NC_008553MeThanoSaeT	CTCAGCTCTTAAA...
AY260439MeThanoArC1	CTCAGCTCTTAAA...
AY260438MeThanoArC1	CTCAGCTCTTAAA...
NC_003901MeThanoArC	CTCAGCTCTTAAA...
NC_003552MeThanoArC	CTCAGCTCTTAAA...
NC_007355MeThanoArC	CTCAGCTCTTAAA...
NC_007955MeThanoCoCC	CTCAGCTCTTAAA...
NC_003551MeThanoPyru	CTCAGCTCTTAAA...
AY714816unCulTuredAr	CTCAGCTCTTAAA...
AY714825unCulTuredAr	CTCAGCTCTTAAA...
AY714837unCulTuredAr	CTCAGCTCTTAAA...
AY714852unCulTuredAr	CTCAGCTCTTAAA...
BX649197unCulTuredAr	CTCAGCTCTTAAA...
AY327048unCulTuredAr	CTCAGCTCTTAAA...
AY714819unCulTuredAr	CTCAGCTCTTAAA...
AY714830unCulTuredAr	CTCAGCTCTTAAA...
AY714870unCulTuredAr	CTCAGCTCTTAAA...

➤ Alignment: to see conserved regions and design primers

# CH<sub>4</sub> formation

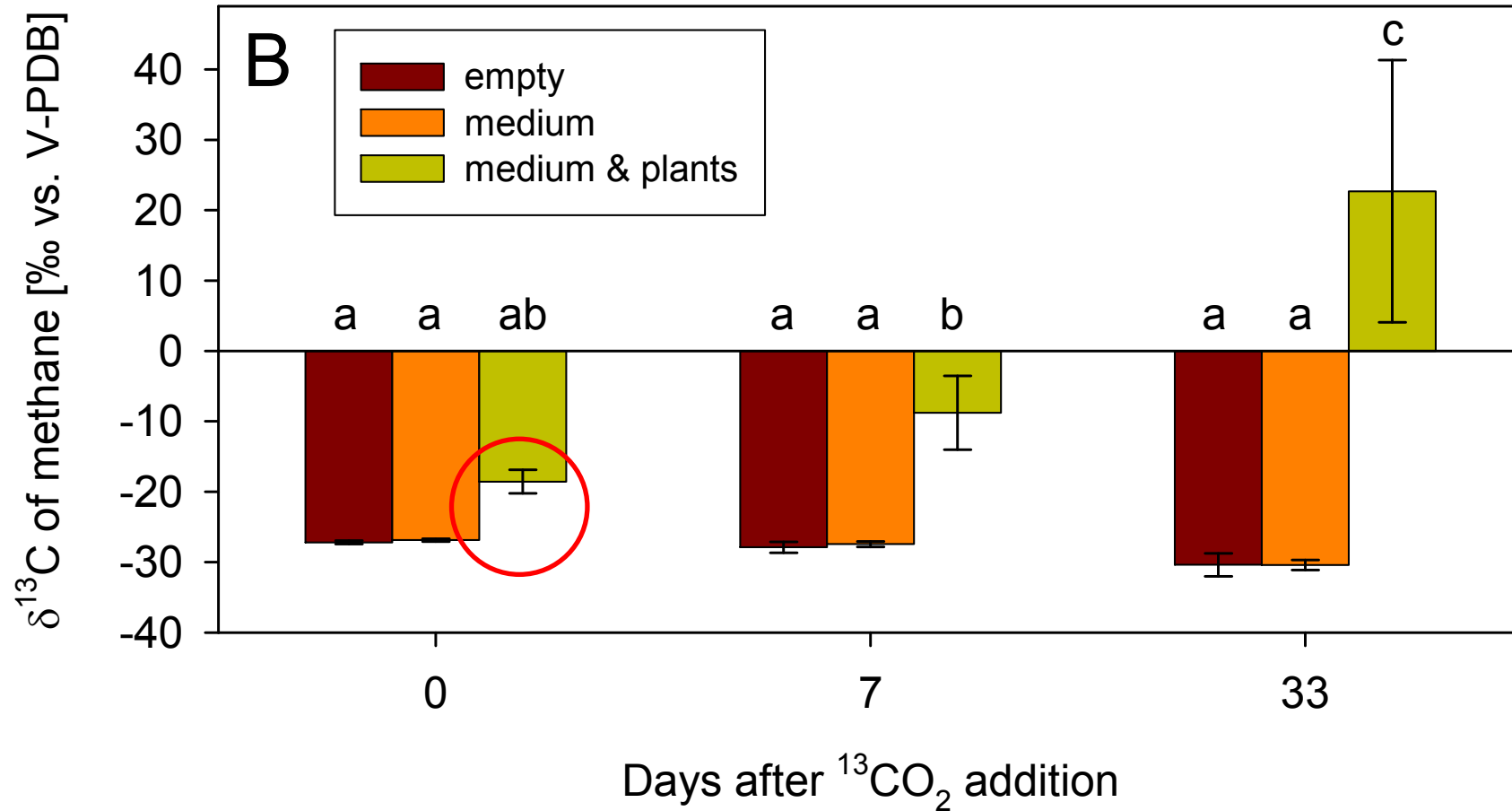


## CH<sub>4</sub> release rate from plants (mean, ± s.e.m.)

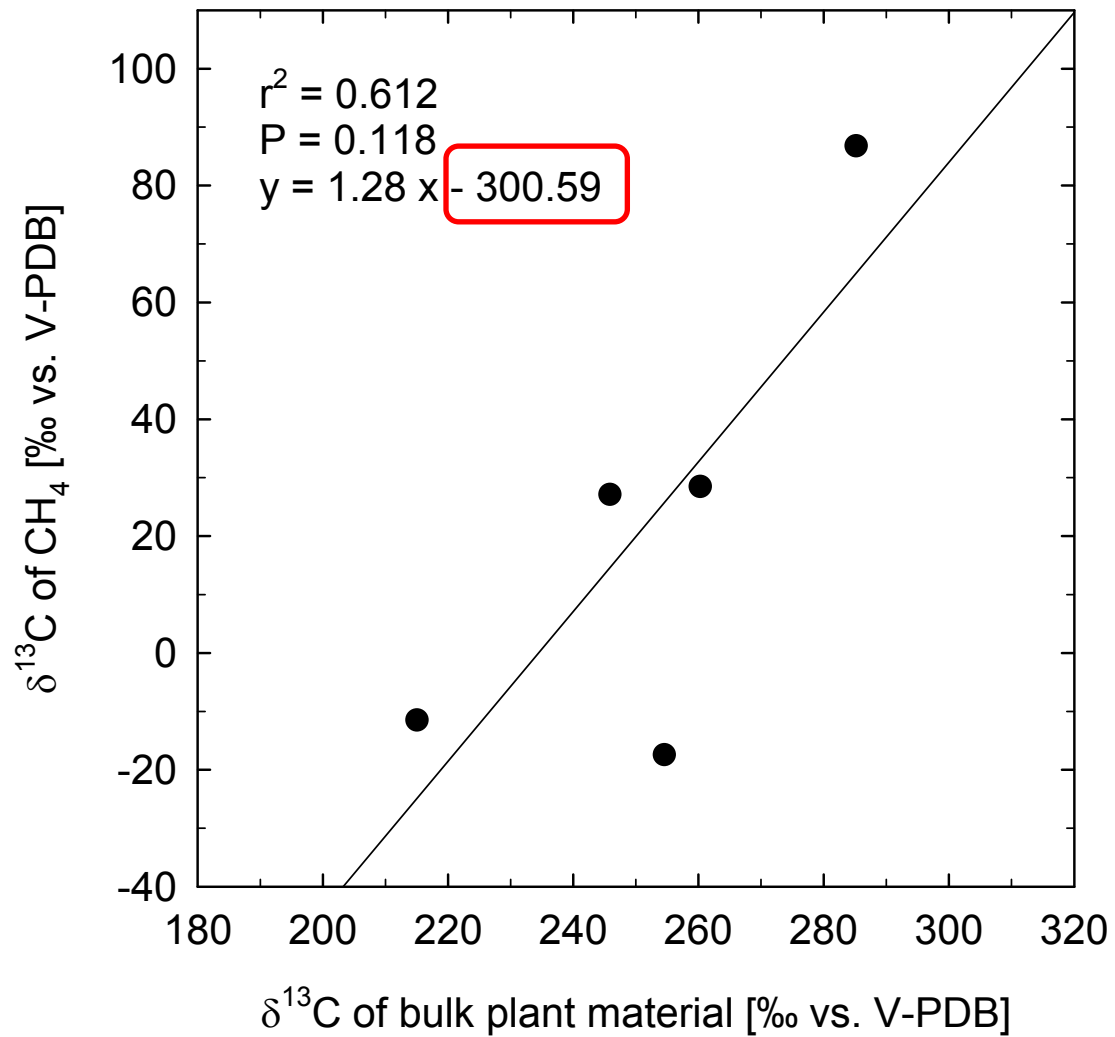
0-7 days: 0.70 ± 0.37 ng g<sup>-1</sup> dry weight h<sup>-1</sup>  
 7-33 days: 0.16 ± 0.11 ng g<sup>-1</sup> dry weight h<sup>-1</sup>  
**0-33 days: 0.24 ± 0.06 ng g<sup>-1</sup> dry weight h<sup>-1</sup>**



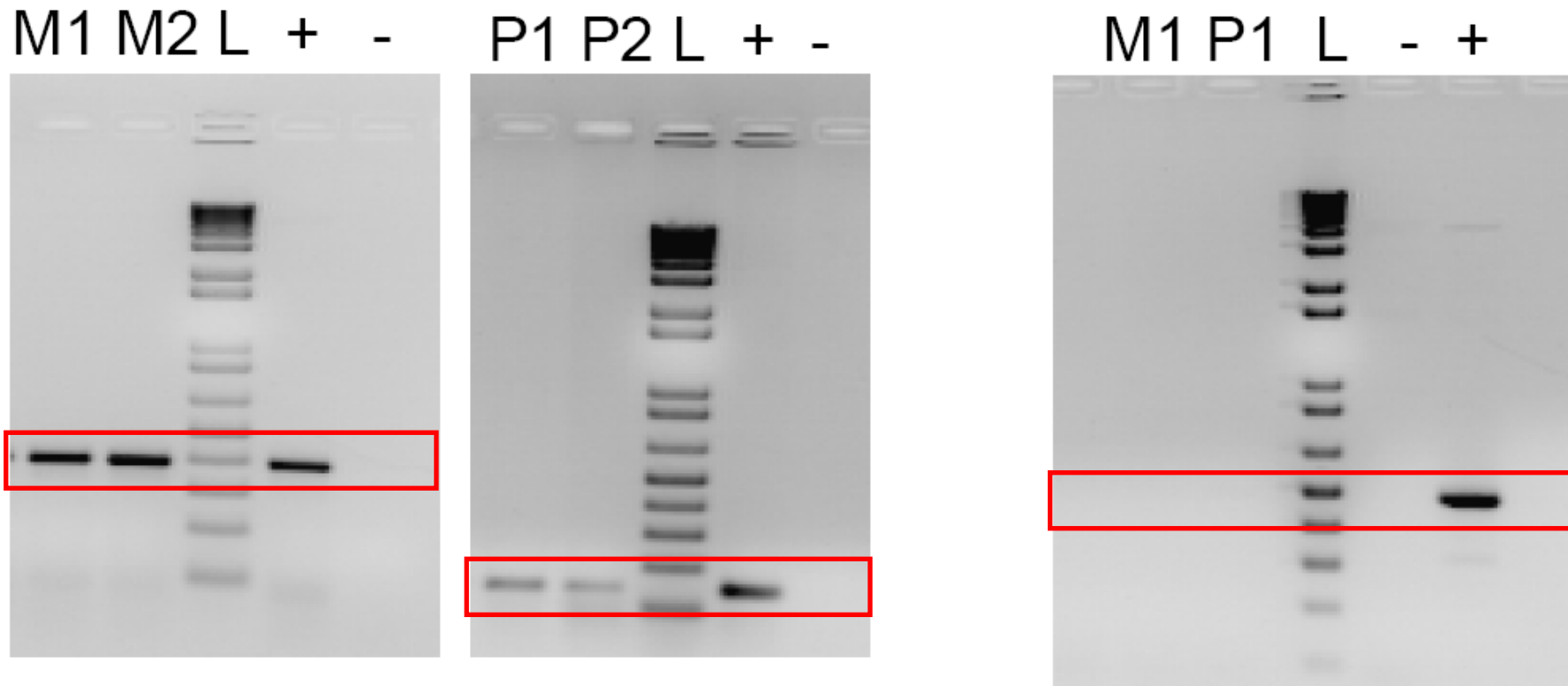
# $\delta^{13}\text{C}$ of $\text{CH}_4$



# Relationship between $\delta^{13}\text{C-CH}_4$ and $\delta^{13}\text{C}$ of bulk plant material



# Electrophoretic analysis of PCR products of medium & plants



***PcPSY***

***PcTUB***

***mcrA***

*PcPSY* = oligonucleitid primer specific for the *Populus × canescens* phytoene synthase gene

*PcTUB* = oligonucleitid primer specific for the *Populus × canescens*  $\beta$ -tubulin gene

*mcrA* = oligonucleitid primer specific for the methyl coenzyme M reductase alpha subunit

# Range of aerobic CH<sub>4</sub> from living and detached plant material



ng CH <sub>4</sub> g <sup>-1</sup> DW h <sup>-1</sup>	References
ND	Kirschbaum & Walcroft, 2008; Nisbet et al., 2009, two species;
0.03	Vigano <i>et al.</i> , 2008, for a fully <sup>13</sup> C-labelled wheat leaf of Dueck et al., 2007, without UV light
<b>0.16–0.7</b>	<b>Our work</b>
0.5–13.5	Wang <i>et al.</i> , 2008, nine emitting species (35 non-emitting species)
–10–42 (not significantly different from 0)	Dueck <i>et al.</i> , 2007, six species
Up to 32	Vigano <i>et al.</i> , 2008, for a fully <sup>13</sup> C-labelled wheat leaf of Dueck <i>et al.</i> , 2007, without UV light
32–49 (not significantly different from 0)	Beerling <i>et al.</i> , 2008, two species
12–370	Keppler <i>et al.</i> , 2006, five species

## Summary

- We have observed release of  $^{13}\text{C}$ -labelled  $\text{CH}_4$  from poplar significantly different from zero under low (UV-free) light conditions after  $^{13}\text{CO}_2$  labelling
- The  $^{13}\text{C}$ -label was detectable in  $\text{CH}_4$  released from the plants already several minutes after start of  $^{13}\text{CO}_2$  labelling
- However, poplar methane emission rates are at the lower end of the reported  $\text{CH}_4$  emission rates from living or detached plant material
- Our work is the first molecular biological proof for the absence of methanogenic microorganisms in plants emitting  $\text{CH}_4$  under aerobic conditions

# The “perfect” aerobic methane experiment?

## Goal:

**Elucidation of CH<sub>4</sub> mechanism(s) with simultaneous determination of realistic emission rates**

- Experiments at ambient gas (CH<sub>4</sub>, O<sub>2</sub>, CO<sub>2</sub>) concentration levels
- Stable isotope labelling essential to differentiate between plant and atmospheric methane
- Analysis of plant-internal reactive oxygen species (ROS)
- Molecular biological verification of the absence of methanogenes
- Application of defined stress situations initiating ROS formation
- ... (open for discussion)