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$_4$-free air

• Headspace was exchanged with synthetic air containing 20% of oxygen and 385 ppm $^{13}$CO$_2$ (99 at% $^{13}$C)

• Flasks were kept in glove box filled with pure N$_2$ 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 μm 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 μmol m\(^{-2}\) s\(^{-1}\) photosynthetic photon flux density (PPFD)
CH$_4$ sampling flowchart

Condensation of sample air with freezing of CO$_2$

Sample volume

CO oxidation to CO$_2$

To sample inlet

Air dryer

Sample inlet

Druckluft

To Precon and IRMS

U-Rohr

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

Forschungszentrum Karlsruhe in der Helmholtz-Gemeinschaft

IMK-IFU Garmisch-Partenkirchen

Nicolas Brüggemann & Jörg-Peter Schnitzler | IMK-IFU | Garmisch

1st workshop on aerobic methane formation, 26/27 Feb 2009, Mainz
**mrcA primers used for PCR**

- **Forward primer:** GGATTCACACARTAYGCWACAGC
- **Reverse primer:** TCATBGCRTAGTTHGGRTAGT

- **Databases:**
  - thousands of mcrA sequences but only "few" are full-length

- **Alignment:** to see conserved regions and design primers
**CH₄ formation**

![Bar chart showing CH₄ mixing ratio over days after ¹³CO₂ addition.]

**Days after ¹³CO₂ addition**

- **0** days: Empty
- **7** days: Medium
- **33** days: Medium + Plants

**CH₄ mixing ratio [ppbv]**

<table>
<thead>
<tr>
<th>Days</th>
<th>CH₄ mixing ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>33</td>
<td>250</td>
</tr>
</tbody>
</table>

**CH₄ release rate from plants (mean, ± s.e.m.)**

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

n = 5, ± SE
Days after $^{13}$CO$_2$ addition

- $\delta^{13}$C of CH$_4$
- empty
- medium
- medium & plants

<table>
<thead>
<tr>
<th>Days</th>
<th>empty</th>
<th>medium</th>
<th>medium &amp; plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>a</td>
<td>a</td>
<td>ab</td>
</tr>
<tr>
<td>7</td>
<td>a</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>33</td>
<td>a</td>
<td>a</td>
<td>c</td>
</tr>
</tbody>
</table>

$\delta^{13}$C of methane [‰ vs. V-PDB]
Relationship between $\delta^{13}$C-CH$_4$ and $\delta^{13}$C of bulk plant material

$r^2 = 0.612$
$P = 0.118$
$y = 1.28x - 300.59$
**Electrophoretic analysis of PCR products of medium & plants**

```
M1  M2  L  +  -  P1  P2  L  +  -  M1  P1  L  -  +
```

**PcPSY**

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

**PcTUB**

PcTUB = oligonucleotide primer specific for the *Populus × canescens* β-tubulin gene

**mcrA**

mcrA = oligonucleotide primer specific for the methyl coenzyme M reductase alpha subunit
## Range of aerobic CH$_4$ from living and detached plant material

<table>
<thead>
<tr>
<th>ng CH$_4$ g$^{-1}$ DW h$^{-1}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>Kirschbaum &amp; Walcroft, 2008; Nisbet et al., 2009, two species;</td>
</tr>
<tr>
<td>0.03</td>
<td>Vigano et al., 2008, for a fully $^{13}$C-labelled wheat leaf of Dueck et al., 2007, without UV light</td>
</tr>
<tr>
<td><strong>0.16–0.7</strong></td>
<td><strong>Our work</strong></td>
</tr>
<tr>
<td>0.5–13.5</td>
<td>Wang et al., 2008, nine emitting species (35 non-emitting species)</td>
</tr>
<tr>
<td>–10–42 (not significantly different from 0)</td>
<td>Dueck et al., 2007, six species</td>
</tr>
<tr>
<td>Up to 32</td>
<td>Vigano et al., 2008, for a fully $^{13}$C-labelled wheat leaf of Dueck et al., 2007, without UV light</td>
</tr>
<tr>
<td>32–49 (not significantly different from 0)</td>
<td>Beerling et al., 2008, two species</td>
</tr>
<tr>
<td>12–370</td>
<td>Keppler et al., 2006, five species</td>
</tr>
</tbody>
</table>
Summary

• We have observed release of $^{13}$C-labelled CH$_4$ from poplar significantly different from zero under low (UV-free) light conditions after $^{13}$CO$_2$ labelling

• The $^{13}$C-label was detectable in CH$_4$ released from the plants already several minutes after start of $^{13}$CO$_2$ labelling

• However, poplar methane emission rates are at the lower end of the reported 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 CH$_4$ under aerobic conditions
The “perfect” aerobic methane experiment?

Goal:
Elucidation of CH4 mechanism(s) with simultaneous determination of realistic emission rates

- Experiments at ambient gas (CH₄, O₂, CO₂) 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)