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# Circadian methane oxidation in the root zone of rice plants

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**Abstract** In the root zone of rice plants aerobic methanotrophic bacteria catalyze the oxidation of CH<sub>4</sub> to CO<sub>2</sub>, thereby reducing CH<sub>4</sub> emissions from paddy soils to the atmosphere. However, methods for in situ quantification of microbial processes in paddy soils are scarce. Here we adapted the push-pull tracer-test (PPT) method to quantify CH<sub>4</sub> oxidation in the root zone of potted rice plants. During a PPT, a test solution containing  $CH_4 \pm O_2$  as reactant(s),  $Cl^-$  and Ar as nonreactive tracers, and BES as an inhibitor of CH<sub>4</sub> production was injected into the root zone at different times throughout the circadian cycle (daytime, early nighttime, late nighttime). After a 2-h incubation phase, the test solution/pore-water mixture was extracted from the same location and rates of CH<sub>4</sub> oxidation were calculated from the ratio of measured reactant and nonreactive tracer concentrations. In separate rice pots, O<sub>2</sub> concentrations in the vicinity of rice roots were measured throughout the circadian cycle using a fiberoptic sensor. Results indicated highly variable CH<sub>4</sub> oxidation rates following a circadian pattern. Mean rates at daytime and early nighttime varied from 62 up to 451  $\mu$ mol l<sup>-1</sup> h<sup>-1</sup>, whereas at late nighttime CH<sub>4</sub> oxidation rates were low, ranging from 13 to 37 µmol  $1^{-1}$  h<sup>-1</sup>. Similarly, daytime O<sub>2</sub> concentration in the vicinity of rice roots increased to up to 250% air saturation, while nighttime O<sub>2</sub> concentration dropped to below detection (<0.15% air saturation). Our results suggest a functional link between root-zone CH<sub>4</sub> oxidation and photosynthetic O<sub>2</sub> supply.

**Keywords** Circadian variation · In situ quantification · Methane oxidation · Oxygen · Push-pull test · Paddy soil

#### Introduction

Methane (CH<sub>4</sub>) is an important greenhouse gas with a warming potential per molecule ~25 times higher than that of carbon dioxide for a calculation period of 100 years (Forster et al. 2007). Among other natural and anthropogenic sources, rice (paddy) soils emit an estimated 31–112 Tg CH<sub>4</sub> a<sup>-1</sup>, thus contributing about 6-18% to global CH<sub>4</sub> emissions (Denman et al. 2007). The uncertainty in emission estimates is partially due to a limited understanding of the factors controlling CH<sub>4</sub> turnover in paddy soils. Thus, better knowledge on the controls of CH<sub>4</sub> dynamics in paddy soils is needed to improve CH<sub>4</sub> emission predictions and to develop more effective mitigation and management strategies (van Bodegom et al. 2001; Wassmann et al. 1993; Yagi et al. 1997).

In paddy soils, CH<sub>4</sub> is produced by methanogenic archaea when soils turn anoxic after flooding (e.g., Kumaraswamy et al. 2000). Generated CH<sub>4</sub> may be

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released to the atmosphere by (1) diffusional transport through the rice plants' aerenchyma, (2) aqueous diffusion to the water table and subsequent partitioning across the air/water interface, and (3) gas-bubble ebullition. Among the three pathways, plant-diffusional transport was found to be dominant in paddy soils (Holzapfel-Pschorn and Seiler 1986; Nouchi et al. 1990; Schütz et al. 1989; Seiler et al. 1983; van der Gon and van Breemen 1993).

However, a substantial fraction of produced CH<sub>4</sub> may never reach the atmosphere due to the activity of aerobic methanotrophic bacteria (e.g., Liesack et al. 2000). These organisms catalyze the oxidation of CH<sub>4</sub> to CO<sub>2</sub>, with CH<sub>4</sub> serving as carbon and energy source, and O<sub>2</sub> being the terminal electron acceptor (Hanson and Hanson 1996). Whereas aerobic methanotrophic bacteria are ubiquitous in paddy soils (Gilbert and Frenzel 1998), aerobic CH<sub>4</sub> oxidation occurs only at oxic/anoxic interfaces, where both O2 and CH4 are present. Therefore, aerobic methanotrophs are most abundant and active at locations such as the soil/water interface and near the root surface in the rice plants' root zone (Bosse and Frenzel 1997; Eller et al. 2005). At the soil/water interface, approximately 80% of the diffusive CH<sub>4</sub> flux (gas-bubble ebullition not included) is oxidized to CO2 (Conrad and Rothfuss 1991; Epp and Chanton 1993; Gilbert and Frenzel 1995). In contrast, balance studies revealed that estimates for CH<sub>4</sub> oxidation in the root zone vary from 0 to 94% of the potential CH<sub>4</sub> flux through the plants' aerenchyma (Chanton et al. 1997), depending on method applied and plant-growth stage. Large variations in the efficiency of root-zone CH<sub>4</sub> oxidation may also be attributed to various factors that are discussed controversially in the literature. Several studies concluded that CH<sub>4</sub> concentration is the ratelimiting factor (Gilbert and Frenzel 1995; van der Gon and Neue 1996). In contrast, availability and competition for O<sub>2</sub> as a control of CH<sub>4</sub> oxidation was proposed by others (King 1996; van Bodegom et al. 2001). Oxygen is delivered to the root zone of rice plants and other aquatic macrophytes by diffusional transport through the plants' aerenchyma and subsequent O<sub>2</sub> leakage from the roots (Armstrong 1964; Calhoun and King 1997; Oremland and Taylor 1977). Reported concentrations of O2 near roots vary dependent on age, species and in which zone measured (Christensen et al. 1994; Frenzel et al. 1992). Furthermore, it was reported that the magnitude of radial  $O_2$  loss from roots of completely submerged rice seedlings was correlated with light (Colmer and Pedersen 2008; Waters et al. 1989).

Different approaches have been employed to measure root-zone CH<sub>4</sub> oxidation, e.g. mass balances between CH<sub>4</sub> production in soil incubations and emission-flux measurements from plants (Bosse and Frenzel 1997; Henckel et al. 2000; Schütz et al. 1989), or CH<sub>4</sub> emission fluxes under inhibited and undisturbed conditions (Epp and Chanton 1993; Krüger et al. 2001). However, the efficiency of inhibitors such as methyl fluoride or difluoromethane, when applied to the headspace of chamber enclosures, strongly depends on the effectiveness of diffusional transport in plants. Similarly, plant incubations under N<sub>2</sub> atmosphere to determine CH<sub>4</sub> emission flux in the absence of CH<sub>4</sub> oxidation may artificially enhance CH<sub>4</sub> production, and thus lead to overestimation of CH<sub>4</sub> oxidation activity when compared to flux measurements in the presence of O<sub>2</sub> (Holzapfel-Pschorn et al. 1985; van der Gon and Neue 1996). Finally, stable isotope measurements (13C/12C ratios and 13Clabeling) were used in several studies to assess CH<sub>4</sub> oxidation in the root zone (Gerard and Chanton 1993; Groot et al. 2003; Krüger et al. 2002). As microbial CH<sub>4</sub> oxidation causes isotope fractionation in the signature of CH<sub>4</sub>, differences in <sup>13</sup>C/<sup>12</sup>C ratios between root-zone CH<sub>4</sub> and CH<sub>4</sub> emitted through rice plants may be used to assess and quantify CH<sub>4</sub> oxidation. However, this approach needs to be used with caution, as additional fractionation due to diffusional transport through the root-shoot transition zone as well as variations in fractionation factors may occur (Butterbach-Bahl et al. 1997; Rao et al. 2008).

Another method for in situ quantification of microbial processes in subsurface environments is the single-well injection-withdrawal test, hereafter referred to as a "push-pull test" (PPT) (Istok et al. 1997). The method consists of injection of an aqueous test solution containing reactant(s) and nonreactive tracer(s) (hereafter referred to as tracer) through a single well at a point of interest, followed by extraction of the test solution/pore water mixture from the same location. An incubation phase after injection may be included to allow for additional reactant turnover. Rate constants may be computed from concentration ratios of reactants (or metabolic products formed) and nonreactive tracers, as measured in samples collected during the PPT's extraction phase



(Haggerty et al. 1998; Schroth and Istok 2006). Pushpull tests were successfully applied in contaminated aquifers to quantify a variety of microbial processes including aerobic respiration, denitrification, sulfate reduction and CH<sub>4</sub> production (e.g., Istok et al. 1997; Kleikemper et al. 2002), as well as reductive dehalogenation (Hageman et al. 2004) and aerobic cometabolism of chlorinated compounds (Kim et al. 2006). In addition, the method was adapted for use in the gaseous phase ("gas push-pull test") to quantify aerobic CH<sub>4</sub> oxidation in the soil vadose zone (e.g., Gomez et al. 2009; Urmann et al. 2005). While the majority of studies interrogated relatively large subsurface volumes by injecting tens to hundreds of liters of test solution, several recent studies demonstrated the utility of PPTs to quantify microbial processes at smaller scales using injection volumes between 10 and 200 ml (Bassein and Jaffe 2009; Koop-Jakobsen and Giblin 2009; Sanders and Trimmer 2006).

The main objective of this study was to adapt PPTs to allow quantification of CH<sub>4</sub> oxidation in the root zone of rice plants under defined conditions in a greenhouse. In particular, we wanted to assess the effect of varying (circadian) O<sub>2</sub> supply through the plants' aerenchyma on CH<sub>4</sub> oxidation activity. In four rice pots, O2 concentrations in the root zone of rice plants were measured using a fiber-optic device to examine circadian O2 dynamics under daytime/nighttime conditions. We performed a series of PPTs in four separate pots under different daytime/nighttime conditions and in the presence/absence of O2 in injected test solutions. Additional PPTs employing <sup>13</sup>CH<sub>4</sub> were performed to support our methodological procedure, while two PPTs (with/without C<sub>2</sub>H<sub>2</sub> as an inhibitor for CH<sub>4</sub> oxidation) were performed to corroborate that apparent CH<sub>4</sub> consumption was microbially mediated.

#### Materials and methods

#### Rice cultivation

Rice plants (*Oryza sativa*, wild type pp309) were cultivated in a greenhouse in 5-l pots using a mixture of one third each (by volume) loamy agricultural soil, quartz sand (0.7–1.2 mm diam.), and dried rice straw that served as fertilizer substitute. In each pot, three rice plants were planted in a triangle. The soil mixture was packed into pots on top of a layer of perlite (0.5 l).

To conduct experiments, rice plants were transferred to a climate chamber operated under similar conditions as the greenhouse: Plants were illuminated for 11 h (hereafter referred to as "daytime", light intensity was  $\sim 20000$  lux just above plants) and kept in dark for 11 h ("nighttime"). Daytime conditions were 28°C and 80% rel. humidity; nighttime conditions were 20°C and 60% rel. humidity. Two 1-hrtransition phases were included to mimic "dawn" and "dusk". To further distinguish nighttime experiments, we will use the term "early nighttime" to refer to experiments beginning shortly after the onset of nighttime, and "late nighttime" for experiments beginning at least 3 h after the onset of nighttime. At the time of the experiments, the plants were in the ripening phase (assessed by visual observation). Grains were mostly hard and yellow colored and pots were completely rooted. Rice shoots were intact and green with some leafs already decaying, which is common at this stage. During all experiments, the water table was maintained at 2-3 cm above the soil surface.

# Root-zone O<sub>2</sub> dynamics

Oxygen concentrations in the vicinity of rice roots were measured using a Fibox-3-Trace fiber-optic  $O_2$  meter in combination with the Trace oxygen dipping probe (Fig. 1, Presens, Regensburg, Germany). Briefly, the method is based on the excitation of dye molecules coated onto a sensor foil at the tip of a

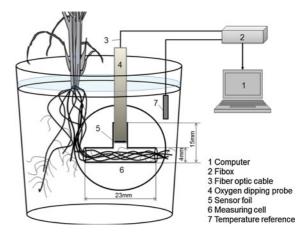


Fig. 1 Oxygen measurement principle employing the fiberoptic  $O_2$  meter. For better visualization the measuring cell is enlarged within the *circle* (i.e., not to scale)



dipping probe by an LED light pulse transmitted through a fiber-optic cable. The resulting luminescence of dye molecules is recorded by the transmitter. In the presence of O<sub>2</sub> this luminescence is quenched, with the quenching effect being proportional to the partial pressure of O<sub>2</sub>. Before measurements a two-point calibration was performed with the software Oxy-View PST3 provided by the manufacturer including automatic temperature compensation. For probe calibration, ambient air as 100% and N<sub>2</sub> gas (Pangas, Dagmarsellen, Switzerland; >99.999 Vol.%) as zero value were used. The probe's detection limit was 0.15% air saturation. Note that in contrast to Clark-type electrodes, no O<sub>2</sub> was consumed during these measurements.

To facilitate  $O_2$  measurements, a fraction of roots of individual rice plants was gently excavated from the soil and carefully rinsed with water. Depending on size, between 5 and 10 washed roots were placed into a

measuring cell (L 23 mm, H 15 mm, ID 4 mm) equipped with the  $\rm O_2$  dipping probe and open on both sides (Fig. 1). Only roots that appeared white or redbrownish (partially encrusted) were used for this purpose, while blackish (fully encrusted) roots were omitted. The measuring cell was then water-saturated and buried 2–3 cm below the soil surface. For four different rice plants,  $\rm O_2$  concentrations were continuously monitored for at least 2 daytime/nighttime cycles using the probe manufacturer's software.

### Push-pull test design

We conducted a total of 20 PPTs in the rice-root zone of five different pots (Table 1). Apart from 14 standard PPTs, four tests were performed using <sup>13</sup>CH<sub>4</sub> (R1–4 <sup>13</sup>C, Table 1), while the final two tests were performed to verify that CH<sub>4</sub> consumption during the tests was microbially mediated (R5, Table 1).

Table 1 Experimental parameters during 20 PPTs performed to quantify  $CH_4$  oxidation in the root zone of rice plants at daytime (D), early nighttime (EN) and late nighttime (LN)

PPT	Time	$O_2$	Injection concentration			BG prior to PPT			
			Cl <sup>-</sup> (mM)	Ar (µM)	CH <sub>4</sub> (μM)	Cl <sup>-</sup> (mM)	Ar (μM)	CH <sub>4</sub> (μM)	
R1a	D	+	2.37	150	167	0.61	5	5	
R2a	D	+	1.95	601	248	0.54	9	21	
R4a	D	+	1.74	573	236	0.49	11	32	
R1b	D	_	2.42	640	306	0.49	37	12	
R2b	D	_	2.1	582	268	0.57	11	18	
R3b	D	_	2.3	621	294	0.43	35	12	
R4b	D	_	2.05	543	255	0.52	12	27	
R1c	LN	+	2.5	231	124	0.78	7	4	
R2c	LN	+	2.01	569	244	0.5	24	5	
R4c	LN	+	2.02	559	241	0.36	26	5	
R1d	EN	_	2.41	609	289	0.39	29	23	
R2d	EN	_	2.49	609	277	0.38	20	6	
R3d	EN	_	2.41	595	281	0.31	28	15	
R4d	LN	_	2.53	632	292	0.35	21	11	
R1 <sup>13</sup> C	D	+	1.66	270	221	0.12	65	46-61	
R2 <sup>13</sup> C	D	+	1.94	275	122	0.4	50	61-71	
R3 <sup>13</sup> C	D	+	1.64	283	251	0.2	66	39-117	
R4 <sup>13</sup> C	D	+	2.04	271	138	0.38	54	53-73	
R5 act.	D	+	2.93	124	91	0.51	3	12	
R5 inhib.	D	+	2.55	77	69	0.28	3	8	

R1-5 represent five different rice pots, a-d represents the sequence of standard PPTs in individual pots, and " $\pm$ " indicates if additional  $O_2$  was supplied during PPTs. Background concentrations (BG) prior to PPTs but after flushing are displayed, except for values of  $^{13}$ C PPTs, which reflect background concentrations of the undisturbed system



In general, to perform a PPT, a customized cylindrical stainless-steel injector (3-cm diam., 3.5cm long, Bopp AG, Zurich, Switzerland) consisting of a 5-layer, sintered wire mesh was installed in each pot  $\sim 2$  cm below the soil surface (Fig. 2). The injector was connected via tygon and Teflon tubing to a Nova System 16-4 piston pump (Encynova, Car-May LLC, Greeley, CO, USA). The dead volume of the system (the volume that could not be pre-flushed with test solution prior to injection) was  $\sim$  29 or 36 ml. Before each PPT, samples for background concentrations of relevant species in pore water were collected in duplicate at the three-way valve closest to the injector. Thereafter, 70 ml of a previously prepared test solution (see below) was injected at a flow rate of  $\sim 10$  ml/min (Fig. 2a). During injection, test-solution samples were collected in duplicate to determine relevant species' injection concentrations (Table 1). After a 2-h incubation phase, 250 ml of the test solution/pore-water mixture was extracted at a flow rate of  $\sim 10$  ml/min and sampled in regular intervals (Fig. 2b). Total test duration was  $\sim 2.5$  h. All samples were subdivided for ion analysis (1 ml) and gas analysis (7 ml injected into 20-ml vials, which were previously sealed with butyl rubber stoppers, crimped, and flushed with N2 gas). Samples for ion analysis were kept frozen until analyzed. Samples for gas analysis were stored at 4°C.

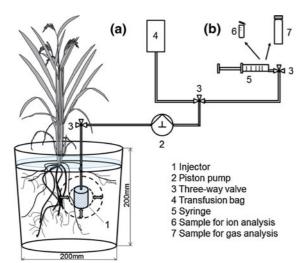


Fig. 2 Simplified scheme of the PPT procedure in the rice-root zone. a Injection phase and b extraction phase. Only one of three rice plants is shown for clarity

# Standard PPTs to quantify CH<sub>4</sub> oxidation

To quantify CH<sub>4</sub> oxidation from standard PPTs, rice pots R1-R4 were flushed prior to injection of test solution with 10 l of anoxic water and additionally with 1 l of anoxic 10 mM 2-bromoethane-sulfonate (BES) solution. Flushing with anoxic water was performed to reduce high and variable CH<sub>4</sub> background concentrations in pore water (Table 1), which had caused difficulties in data analyses of preliminary PPTs (not shown). Flushing with BES was done to inhibit CH<sub>4</sub> production; the effective concentration range of BES for inhibition of CH<sub>4</sub> production was inferred from preliminary batch experiments (not shown). Test solutions for standard PPTs consisted of 2 mM Cl<sup>-</sup> as ionic tracer and 10 mM BES dissolved in deionized water, which was subsequently sparged for at least 35 min with a gas mixture consisting of 50 vol.% Ar as dissolved-gas tracer and 30% CH<sub>4</sub>/20% O<sub>2</sub> as reactants, or, 50% Ar, 30% CH<sub>4</sub> and 20% N<sub>2</sub> for experiments without external O<sub>2</sub> supply (Table 1). Test solutions were subsequently transferred to transfusion bags (Macopharma, Mouvaux, France) and kept under water to minimize gas exchange.

# Verification of standard PPT procedure and microbial CH<sub>4</sub> oxidation

To assess the effect of extensive flushing of rice pots on methanotrophic activity, four PPTs utilizing <sup>13</sup>CH<sub>4</sub> were performed in rice pots R1–R4 prior to standard PPTs (Table 1). In <sup>13</sup>C PPTs extensive flushing was unnecessary due to the low natural abundance of <sup>13</sup>C in background pore water. Instead, rice pots were only flushed with 1 l of anoxic 10 mM BES solution prior to PPTs to inhibit CH<sub>4</sub> production. Test solutions were prepared in similar fashion as before, however, without addition of CH<sub>4</sub> to the sparged gas mixture. Rather, 5 ml of <sup>13</sup>CH<sub>4</sub> gas (99 atom% <sup>13</sup>C, Isotec <sup>™</sup>, Miamisburg, Ohio, USA) was directly injected into transfusion bags and equilibrated with the test solutions.

Finally, two additional PPTs were performed in a separate rice pot (R5, Table 1) to verify that  $CH_4$  consumption during the tests was microbially mediated. The first PPT (R5 act.) was performed in similar fashion as described above for standard PPTs. For the second PPT (R5 inhib.), 5%  $C_2H_2$ , an inhibitor for  $CH_4$ 



oxidation (Yoshinari and Knowles 1976), was additionally added to the sparged gas mixture.

#### Analytical methods

Chloride and BES were measured on a DX-320 ionchromatography system (Dionex, Sunnyvale, CA, USA) as described by Kleikemper et al. (2002). Methane and C<sub>2</sub>H<sub>2</sub> were quantified using a gas chromatograph (Trace GC Ultra, Thermo Fisher Scientific, Rodano, Italy) equipped with a Porapak-N column at 85°C and an FID detector. Carrier gas was N<sub>2</sub>. Argon was measured on a gas chromatograph (Trace GC Ultra, Thermo Fisher Scientific, Rodano, Italy) equipped with a TCD detector and a 5A-Molsieve column (10-m long, 2-mm i.d.) at 35°C with a back-flushed pre-column to remove CO2 and H2O (Gonzalez-Gil et al. 2007). Carrier gas was N2. Analysis of <sup>13</sup>CH<sub>4</sub> (for PPTs R1-4 <sup>13</sup>C) was performed on a Trace GC Ultra (Thermo Fisher Scientific, Rodano, Italy) equipped with a 5A—Molsieve column (PLOT fused silica, 25-m long, 0.32-mm i.d., CP7536, Varian) with hydrogen as carrier gas at 30°C and coupled to a DSQ mass spectrometer (Thermo Fisher Scientific, Rodano, Italy). Quantification was accomplished using the Xcalibur software package (Thermo Fisher Scientific Inc., USA).

Measured gas-phase concentrations of  $CH_4$  and Ar were converted to aqueous-phase (dissolved) concentrations using respective Henry constants at  $20^{\circ}C$  (in atm/mol: 37600 ( $CH_4$ ) and 36960 (Ar) (Sander 1999)) employing the calculation method of Kampbell and Vandegrift (1998).

#### Estimation of kinetic parameters

To generate breakthrough curves (BTCs) and for subsequent estimation of kinetic parameters, measured concentrations for  $Cl^-$ ,  $CH_4$  and Ar were converted to relative concentrations ( $C^*$ ) by dividing concentrations from extraction samples by the respective injection concentration after correction for background concentration contained in pore water. In this fashion, relative  $Cl^-$  concentrations ( $C_{Cl}^*$ ) were computed using (Kim et al. 2006):

$$C_{\text{Cl}}^* = (C_{\text{Cl}} - C_{\text{Cl,bg}}) / (C_{\text{Cl,inj}} - C_{\text{Cl,bg}})$$
 (1)

where  $C_{\text{Cl}}$  is  $\text{Cl}^-$  concentration in an extraction sample,  $C_{\text{Cl,bg}}$  is background concentration in pore

water, and  $C_{\text{Cl,inj}}$  is injection concentration. Here,  $C_{\text{Cl}}^*$  provides a measure of dilution between injected test solution and background pore water. It was subsequently used to correct relative CH<sub>4</sub> ( $C_{\text{CH}_4}^*$ ) and Ar ( $C_{\text{Ar}}^*$ ) concentrations according to (Nauer and Schroth 2010):

$$C_{\text{CH}_4}^* = \left[ C_{\text{CH}_4} - \left( 1 - C_{\text{Cl}}^* \right) C_{\text{CH}_4,\text{bg}} \right] / C_{\text{CH}_4,\text{inj}}$$
 (2)

$$C_{\text{Ar}}^* = \left[ C_{\text{Ar}} - \left( 1 - C_{\text{Cl}}^* \right) C_{\text{Ar,bg}} \right] / C_{\text{Ar,inj}}$$
 (3)

where  $C_{\text{CH}_4}$  and  $C_{\text{Ar}}$  are CH<sub>4</sub> and Ar concentrations in extraction samples,  $C_{\text{CH}_4,\text{bg}}$  and  $C_{\text{Ar},\text{bg}}$  are their background concentrations in pore water, and  $C_{\text{CH}_4,\text{inj}}$  and  $C_{\text{Ar},\text{inj}}$  are injection concentrations. For <sup>13</sup>CH<sub>4</sub>, background concentration was considered negligible compared to the amount added during <sup>13</sup>C PPTs, thus no correction for these data was implemented. Breakthrough curves for different compounds were then obtained by plotting  $C^*$  versus relative extracted volume (i.e. extracted volume  $V_{\text{ext}}$  divided by total injected volume  $V_{\text{inj}}$  (Istok et al. 1997)).

To determine apparent rate coefficients k (h<sup>-1</sup>) for CH<sub>4</sub> oxidation, we plotted for each sample j the natural logarithm of  $C_{\text{CH}_4}^*/C_{\text{Ar}}^*$  against computed residence time ( $t_R$ ), thus accounting for partial consumption of CH<sub>4</sub> during PPTs' injection and incubation phases (Schroth and Istok 2006):

$$\ln\left(\frac{C_{\text{CH4}}^*}{C_{\text{Ar}}^*}\right)_j = kt_{R,j} \tag{4}$$

with

$$t_{R,j} = t_j^* + \frac{\int_{t_{\text{ext}}=0}^{t_{\text{ext}}} Q_{\text{ext}} C_{\text{Cl,corr}}(t) dt}{M_{\text{Cl}}} T_{\text{inj}}$$
 (5)

where  $t^*$  is time since the end of injection,  $t_{\rm ext}$  is time since extraction began,  $Q_{\rm ext}$  is extraction flow rate,  $C_{\rm Cl,corr}$  is background-corrected Cl<sup>-</sup> concentration,  $M_{\rm Cl}$  is the total mass of Cl<sup>-</sup> injected, and  $T_{\rm inj}$  is injection time. Estimates for k were obtained by fitting Eq. 4 to quasi-linear segments of experimental data using linear regression. The first five data points of each test were omitted from fitting, as they represented the system's dead volume. Note that while Cl<sup>-</sup> was used to account for dilution of test solution with background pore water during PPTs, Ar was employed for rate calculations because of its similar physical transport properties compared to CH<sub>4</sub> (diffusion coefficients in air at 25°C in cm<sup>2</sup>/s: 0.19 for Ar



(calculated according to Fuller et al. (1966)); 0.23 for CH<sub>4</sub> (Massman 1998); Henry constants see above). Thus, Ar was used to account for possible dissolved CH<sub>4</sub> losses due to partitioning into trapped gas bubbles, diffusional plant transport, and outgassing across the air/water interface. The diffusion coefficients of tracers and reactants in water (at 25°C in cm<sup>2</sup>/s) are  $1.98 \times 10^{-5}$  for Cl<sup>-</sup> (Lobo et al. 1998),  $1.88 \times 10^{-5}$  for CH<sub>4</sub> (Witherspoon and Saraf 1965),  $1.90 \times 10^{-5}$  for Ar (Yaws 2010), and  $2.20 \times 10^{-5}$  for O<sub>2</sub> (Ferrell and Himmelblau 1967).

Absolute rates of  $CH_4$  oxidation (µmol  $I^{-1}$   $h^{-1}$ ) were determined by multiplying k estimates from individual data segments by the corresponding average  $CH_4$  concentration or by the minimum and maximum  $CH_4$  concentration observed in respective segments. In this fashion, a range of  $CH_4$  oxidation rates was obtained for each PPT. Note that for the calculation of rates derived by  $^{13}C$  PPT, total  $CH_4$  concentration, i.e. the sum of  $^{13}C$ - $CH_4$  and  $^{12}C$ - $CH_4$  background concentration, was used.

#### Results

# Root-zone O<sub>2</sub> dynamics

Oxygen concentrations near the roots of all four rice plants showed a circadian pattern with increasing O<sub>2</sub> concentrations at daytime and decreasing O2 concentrations to below detection at nighttime (Fig. 3). This pattern was similar for all plants; however, the magnitude of O<sub>2</sub> concentrations differed between plants with peak O<sub>2</sub> concentrations during daytime ranging between 90 and 250% air saturation. All plants showed a rapid change in O2 concentrations in response to light conditions, but with some delay with respect to the onset of dawn and dusk. This delay was most pronounced for the rice plant shown in Fig. 3b, where O<sub>2</sub> concentrations near the roots began to increase only about 6 h after the onset of dawn. Small fluctuations at the beginning of measurements (Fig. 3c, d) may have arisen from the time needed to equilibrate the probe after emplacement.

# Push-pull test performance

In all PPTs, BTCs for Cl<sup>-</sup> showed a continuous decline in C\*, indicating that test solution was

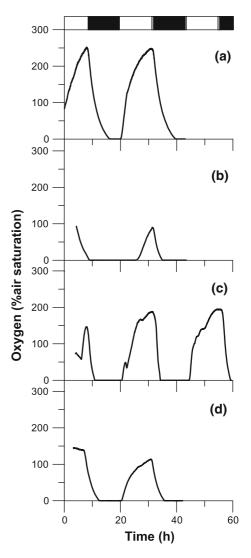
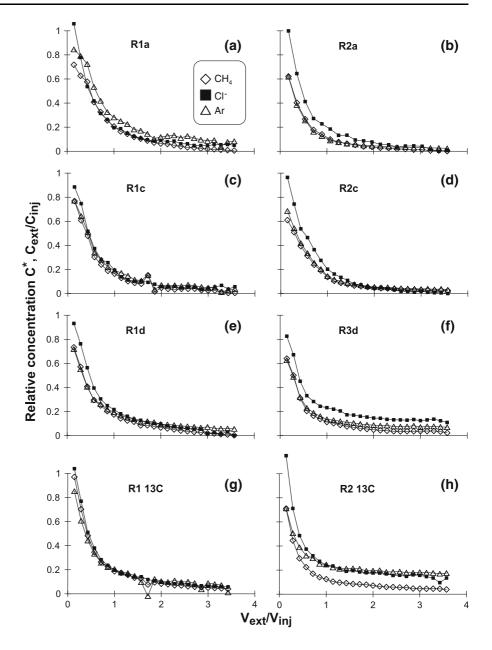


Fig. 3 Root  $O_2$  dynamics in four different rice plants (*Oryza sativa*). Night (*black bar*), day (*open bar*), transition zone (*grey bar*). Note that measurements in a, b and d were terminated at  $t \cong 42 \text{ h}$ 

increasingly diluted with background pore water during the PPTs' extraction phase (Fig. 4). Initially the decline was fast, but this was followed by a slower decline during later stages of extraction. Breakthrough curves for Ar and CH<sub>4</sub> generally exhibited a similar pattern. However, in most cases relative Cl<sup>-</sup> concentrations were higher than relative Ar concentrations indicating that some Ar was lost from the system in addition to the dilution accounted for by Cl<sup>-</sup>. Towards the end of extraction, relative Ar and Cl<sup>-</sup> concentrations leveled off to a nearly constant value, which was used for background correction when the originally



Fig. 4 Selected extraction breakthrough curves showing relative concentrations ( $C^*$ ) of CH<sub>4</sub>, Cl<sup>-</sup> and Ar for standard PPTs conducted at daytime (**a**, **b**), late nighttime (**c**, **d**) and early nighttime (**e**, **f**), and for daytime <sup>13</sup>C-PPTs (**g**, **h**)



measured background value was higher than this value. Finally, relative  $CH_4$  concentrations were usually smaller than relative Ar concentrations, in particular during later stages of extraction (Fig. 4). This was considered to be indicative of  $CH_4$  consumption.

Mass recovery of Cl<sup>-</sup>, Ar and CH<sub>4</sub> was computed from respective BTCs for those data segments that were subsequently employed for the calculation of rate constants. Early-time data points were omitted from these calculations (system dead volume), thus values

of relative mass recovered (total mass recovered/total mass injected  $\times$  100%) were always substantially smaller than 100% (Table 2). Relative mass recoveries of Cl $^-$  (22–65%) were commonly higher than for Ar (13–60%), while CH<sub>4</sub> usually exhibited the smallest relative mass recoveries (5–30%). A slightly higher mass recovery for CH<sub>4</sub> compared to Ar was obtained in the inhibition test (R5 inhib., Table 2), whereas a substantially lower mass recovery for CH<sub>4</sub> was obtained in R5 act., indicating CH<sub>4</sub> consumption during the latter test. Note that for calculation of rate



**Table 2** Estimates of rate constants k (with 95% confidence intervals  $2\sigma_k$ ) for two individual data segments of 20 PPTs, mean CH<sub>4</sub> oxidation rates for data segments, and relative mass recovery of Cl<sup>-</sup>, Ar and CH<sub>4</sub>

PPT	Time	Segment 1 (early	y-time)	Segment 2 (late-	Relative mass recovery			
		$k \pm 2\sigma_{\kappa} (h^{-1})$	Mean rate (μmol l <sup>-1</sup> h <sup>-1</sup> )	$k \pm 2\sigma_{\kappa} (h^{-1})$	Mean rate (μmol l <sup>-1</sup> h <sup>-1</sup> )	Cl <sup>-</sup> (%)	Ar (%)	CH <sub>4</sub> (%)
R1a	D	$1.1 \pm 0.98$	84	$7.9 \pm 1.26$	185	31	46	25
R2a	D	$3.5 \pm 0.59$	99	$13.5 \pm 8.63$	232	24	15	13
R4a	D	$1.3 \pm 0.86$	67	$7.0 \pm 2.47$	244	41	20	25
R1b	D	$1.9 \pm 0.41$	109	$8.4 \pm 2.20$	315	32	25	19
R2b	D	$3.1 \pm 1.90$	96	$21.6 \pm 4.58$	359	32	13	11
R3b	D	$2.3 \pm 0.15$	62	$6.1 \pm 1.10$	96	43	24	14
R4b	D	$2.7 \pm 0.91$	129	$15.1 \pm 6.37$	451	48	21	23
R1c	LN	$2.0 \pm 0.83$	37	n.a. <sup>a</sup>	n.a. <sup>a</sup>	27	29	21
R2c	LN	$0.7 \pm 0.15$	13	n.a. <sup>a</sup>	n.a. <sup>a</sup>	23	22	19
R4c	LN	$-0.5 \pm 0.19^{b}$	-11	n.a. <sup>a</sup>	n.a. <sup>a</sup>	26	26	26
R1d	EN	$2.2 \pm 0.49$	134	$8.4 \pm 1.82$	82	30	33	23
R2d	EN	$4.0 \pm 0.21$	93	n.a. <sup>a</sup>	n.a. <sup>a</sup>	57	20	14
R3d	EN	$2.5 \pm 0.23$	81	n.a. <sup>a</sup>	n.a. <sup>a</sup>	48	29	18
R4d	LN	$0.5 \pm 0.11$	20	n.a. <sup>a</sup>	n.a. <sup>a</sup>	56	25	31
R1 13C	D	$1.9 \pm 0.45$	95	n.a. <sup>a</sup>	n.a. <sup>a</sup>	34	30	30
R2 <sup>13</sup> C	D	$2.2 \pm 0.19$	37	n.a. <sup>a</sup>	n.a. <sup>a</sup>	54	60	23
R3 <sup>13</sup> C	D	$0.8 \pm 0.52$	25	n.a. <sup>a</sup>	n.a. <sup>a</sup>	22	24	26
R4 <sup>13</sup> C	D	$8.2 \pm 2.94$	292	n.a. <sup>a</sup>	n.a. <sup>a</sup>	42	31	27
R5 act.	D	$3.7 \pm 0.98$	13	$6.4 \pm 1.3$	11	65	15	05
R5 inhib.	D	$-0.3 \pm 0.41^{b}$	-2	n.a. <sup>a</sup>	n.a. <sup>a</sup>	42	28	34

Early-time data (first 5 data points) were omitted from calculations, as they represented the system's dead volume

constants high mass recovery is not a prerequisite (Haggerty et al. 1998).

# Standard PPTs to quantify CH<sub>4</sub> oxidation

Several data sets exhibited a curved rather than a linear decrease in  $\ln(C_{\text{CH}_4}^*/C_{\text{Ar}}^*)$  during extraction, an example is shown in R4b data (Fig. 5). We chose to process curved PPT data sets in two quasi-linear segments, thus yielding two k values (Table 2). In standard PPTs, smaller k values ranging from 0.5–4 h<sup>-1</sup> were obtained for early-time data when absolute CH<sub>4</sub> concentrations were high, while k values for late-time data (when CH<sub>4</sub> concentrations were small) ranged from 6.1–21.6 h<sup>-1</sup>.

In general, calculated mean rates showed a high variability especially at daytime and early nighttime (Table 2). PPTs conducted at late nighttime resulted

either in low rates of  $CH_4$  oxidation or even in slight production of  $CH_4$  (negative rates in Table 2; R4c data set in Fig. 5). Adding  $O_2$  to the injection solution had little effect on rate constants and  $CH_4$  oxidation rates (Table 2). In those experiments,  $O_2$  concentration declined rapidly to below detection early during extraction (data not shown), indicating that  $O_2$  was rapidly consumed in the soil.

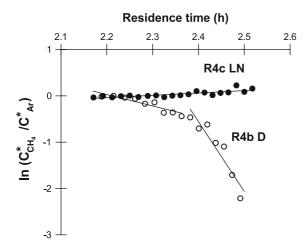
Verification of standard PPT procedure and microbial CH<sub>4</sub> oxidation

Daytime PPTs with <sup>13</sup>CH<sub>4</sub> were conducted to test if flushing before standard PPTs had any adverse effects on CH<sub>4</sub> oxidation. Breakthrough curves similar to those of standard PPTs were obtained for <sup>13</sup>C PPTs (examples in Fig. 4g, h). Rate plots for all <sup>13</sup>C PPTs exhibited one quasi-linear segment (not shown), from



 $<sup>^{</sup>a}$  n.a.: no regression line was fitted for segment 2, as data exhibited a single slope (i.e., one k value)

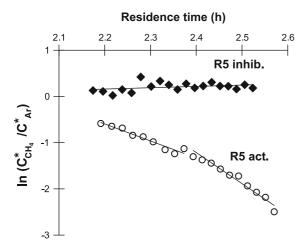
<sup>&</sup>lt;sup>b</sup> Production of methane is indicated with a (-) sign



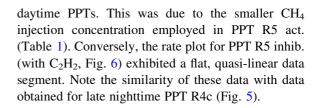
**Fig. 5** Plot to determine rate constants *k* for PPTs conducted during late nighttime (LN) and daytime (D) in rice pot R4

which a single rate constant was computed for each test (Table 2). The resulting k values ranged between 0.8 and 8.2 h<sup>-1</sup> (Table 2). Calculated rates were in the range of daytime standard PPTs ranging from 25 up to 292  $\mu$ mol l<sup>-1</sup> h<sup>-1</sup> (Table 2), also exhibiting substantial variability.

The rate plot for PPT R5 act. (without  $C_2H_2$ , Fig. 6) exhibited a pattern similar to daytime standard PPTs, displaying two quasi-linear data segments. Consequently, calculated k values (3.7 and 6.4 h<sup>-1</sup>, Table 2) were in the same range as for daytime PPTs. However, the computed  $CH_4$  oxidation rate (13  $\mu$ mol  $I^{-1}$  h<sup>-1</sup>, Table 2) was substantially lower than rates of other



**Fig. 6** Plot to determine rate constants k for PPT R5 act. (without  $C_2H_2$ ), and PPT R5 inhib. (with  $C_2H_2$  to inhibit  $CH_4$  oxidation)



#### Discussion

Root-zone O<sub>2</sub> dynamics

Data presented here (Fig. 3) support previous findings in that O<sub>2</sub> concentrations increased in the root zone during daytime and decreased at nighttime, thus following a circadian pattern (Frenzel et al. 1992; Waters et al. 1989). The increase of  $O_2$  concentration was indicative of O<sub>2</sub> production coupled to diffusional transport through the plants' aerenchyma, whereas a decrease might be caused by root respiration, and/or chemical and microbial O<sub>2</sub> consumption in rice soil. A noticeable delay in O<sub>2</sub> concentration increase at dawn (Fig. 3b) was possibly due to root/shoot junction resistance (van der Gon and van Breemen 1993), which would have increased the time necessary for the buildup of a sufficiently large O<sub>2</sub> concentration gradient. Moreover, the time required for  $O_2$  to diffuse from roots surfaces to the  $O_2$  probe might have created an additional delay. This delay, however, we would expect to be similar in magnitude for all O<sub>2</sub> measurements.

Peak O<sub>2</sub> concentrations in our study (250% air saturation in Fig. 3a corresponds to  $688 \mu M O_2$ dissolved in water) were highly variable and substantially higher compared to other studies measuring O<sub>2</sub> availability in rice soil (10 to 150 µM O<sub>2</sub> (Frenzel et al. 1992), and 0 to 96 µM in dim light (Revsbech et al. 1999)). This might be caused by several factors, including a lower O<sub>2</sub> demand in the vicinity of the roots, as roots were separated from bulk soil by the measuring cell. Bulk soil (reduced conditions) is usually a major sink for O<sub>2</sub>. In addition, we noted patchy red-brownish precipitates on root surfaces, which may be indicative of a heterogeneous  $O_2$ distribution. The precipitates were likely iron oxides, which are commonly found on rice roots (Chen et al. 1980; Macfie and Crowder 1987). Furthermore, O<sub>2</sub> concentration in the root zone may also be influenced by plant-growth conditions (Colmer et al. 1998) and

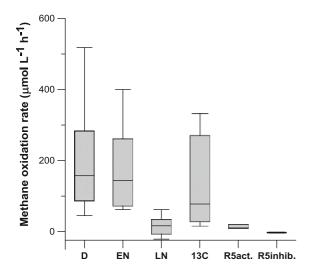


by the roots' physiological condition (Colmer 2003). Thus, having used roots of healthy appearance for our measurements (omitting decaying, blackish and fully encrusted roots) may explain in part the high  $\rm O_2$  concentrations observed.

Nonetheless, both the distinct circadian pattern and the magnitude of  $O_2$  concentration were a clear indication that photosynthetic activity in the plant canopy was an important factor for  $O_2$  delivery to the root zone, as maximum  $O_2$  concentrations near the roots substantially exceeded atmospheric  $O_2$  saturation (Fig. 3).

# Circadian pattern of CH<sub>4</sub> oxidation

Methane oxidation rates obtained at daytime were considerably higher than at late nighttime (Fig. 7), which is in agreement with the measured circadian pattern of O<sub>2</sub> concentration. During early nighttime, O<sub>2</sub> was apparently still supplied to the root zone, as seen from the gradual decline in O<sub>2</sub> concentration at the onset of nighttime (Fig. 3). Consequently, CH<sub>4</sub> oxidation rates obtained at early nighttime were similar to daytime rates and thus substantially higher than at late nighttime (Fig. 7). At late nighttime, we



**Fig. 7** Box-whisker plot for comparison of maximum and minimum  $CH_4$  oxidation rates obtained from PPTs conducted at daytime (D), early nighttime (EN), and late nighttime (LN), and for  $^{13}C$  PPTs (daytime) and PPTs R5 act. (without  $C_2H_2$ ) and R5 inhib. (with  $C_2H_2$ ). *Boxes* reflect 25 and 75 percentiles, *horizontal lines* show median values, whiskers show maximum and minimum values. Note that PPT R5 inhib. yielded only a single data point

detected either low CH<sub>4</sub> oxidation rates or even slight production of CH<sub>4</sub> (Fig. 7; Table 2). Possibly, CH<sub>4</sub> was produced in regions of the rice soil where BES was not entirely efficient in inhibiting CH<sub>4</sub> production. Nonetheless, our data suggest that CH<sub>4</sub> oxidation at daytime was largely fuelled by photosynthetically produced O2. At late nighttime, low rates of CH4 oxidation clearly indicated an O2 limitation, as similar CH<sub>4</sub> injection concentrations were employed in all standard PPTs (Table 1). Our results are in general agreement with several previous studies (e.g., Oremland and Taylor 1977), but they contrast results obtained by Van der Nat et al. (1998), who employed the CH<sub>3</sub>F inhibition flux chamber technique and found little effect of light conditions on CH<sub>4</sub> oxidation in the root zone of wetland plants Phragmites australis and Scirpus lacrustris.

To allow a comparison of CH<sub>4</sub> oxidation rates obtained in this study with literature values, we converted our data to units of umol g(dry weight,  $(d.w.)^{-1} h^{-1}$  using an estimated porosity of 0.52 (loamy sand) and assuming water-saturated conditions. Rates calculated for standard PPTs' segment 1 (Table 2) ranged from 0.01 to 0.56  $\mu$ mol g(d.w.)<sup>-1</sup> h<sup>-1</sup>. They were in the range of data reported for shortterm soil-slurry incubations of rhizospheric rice soil  $(0.01-0.2 \mu \text{mol g(d.w.})^{-1} \text{ h}^{-1} \text{ (Henckel et al. 2000))},$ but were lower than rates obtained after long-term incubation (up to 2 µmol g(d.w.)<sup>-1</sup> h<sup>-1</sup> (Bosse and Frenzel 1997; Eller and Frenzel 2001), and higher in comparison to bulk soil incubations (<0.01 µmol g<sup>-1</sup> h<sup>-1</sup>, no indication if dry or wet weight (Wang et al. 1997)). Conversely, higher rates (up to 9 µmol g(d.w.)<sup>-1</sup> h<sup>-1</sup>) were calculated for standard PPTs' segment 2 (Table 2), which may have been the result of increased O<sub>2</sub> availability and longer reaction/contact time of that portion of test solution in the root zone (due to longer travel distance (Haggerty et al. 1998)). In general, high variability of calculated in situ CH<sub>4</sub> oxidation rates was considered an indication for the system's heterogeneity.

Verification of standard PPT procedure and microbial CH<sub>4</sub> oxidation

Our study revealed only slight differences in rate constants and rates of CH<sub>4</sub> oxidation between daytime standard and <sup>13</sup>C-PPTs (Table 2; Fig. 7). Hence, <sup>13</sup>C-PPTs indicated that extensive flushing prior to



injection of test solution during standard PPTs had little adverse effects on measured CH<sub>4</sub> oxidation rates. Indeed, a slightly higher variability in rate constants and CH<sub>4</sub> oxidation rates in <sup>13</sup>C-PPTs compared to daytime standard PPTs was noticeable (Table 2), which might indicate that flushing reduced adverse effects of variable background concentrations in standard PPTs. While <sup>13</sup>C-PPTs allowed performing tests with less disturbance (less flushing) to the system, standard PPTs required less complex analytical methods (no mass spectrometry required); the latter are therefore more cost effective for routine applications.

Finally, we verified that CH<sub>4</sub> oxidation was microbially mediated by conducting an active test followed by an inhibition test. Results clearly showed that CH<sub>4</sub> was consumed during PPT R5 act., whereas CH<sub>4</sub> oxidation was effectively inhibited by C<sub>2</sub>H<sub>2</sub> during PPT R5 inhib (Figs. 6, 7). Similar to late nighttime PPTs, a slight production of CH<sub>4</sub> during the active test may indicate that BES was not entirely efficient in inhibiting CH<sub>4</sub> production. However, as a result of BES added to the PPTs' test solutions CH<sub>4</sub> production was low in magnitude compared to CH<sub>4</sub> oxidation, e.g., in daytime PPTs. Consequently, any underestimation of CH<sub>4</sub> oxidation rates as a result of concurrent CH<sub>4</sub> production was expected to be small.

# Conclusions

We successfully adapted the PPT method to quantify CH<sub>4</sub> oxidation in situ in the root zone of rice plants. In these PPTs, we employed both ionic and dissolved-gas tracers to account for dilution with background pore water as well as dissolved-gas transport phenomena. Our results indicated that CH<sub>4</sub> oxidation followed a circadian pattern. This suggests that CH<sub>4</sub> oxidation was mainly limited by  $O_2$  availability in the rice-root zone, which was supported by our O2 concentration measurements. Extensive flushing prior to standard PPTs to reduce CH<sub>4</sub> background concentrations in pore water appeared to have little adverse effect on measured CH<sub>4</sub> oxidation rates, as was confirmed in separate PPTs employing <sup>13</sup>CH<sub>4</sub>. With further adaptation, the presented methodology may be used to quantify a variety of processes in situ in the root zone of plants in waterlogged habitats, e.g. wetlands.

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