Technical note: Methionine, a precursor of methane in living plants

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INTRODUCTION

The observation that plants produce methane (CH_4) under aerobic conditions has caused considerable controversy amongst the scientific community (Keppler et al., 2006;Dueck et al., 2007;Evans, 2007;Beerling et al., 2008;Kirschbaum and Walcroft, 2008;McLeod et al., 2008;Vigano et al., 2008;Wang et al., 2008;Nisbet et al., 2009;Bloom et al., 2010, Covey et al. 2012, Zhang et al. 2014). Despite much scepticism a number of recent studies provide support for the phenomenon not only for living plants but also for dead plant matter (Keppler et al., 2009;Qaderi and Reid, 2011;Bruhn et al., 2012;Wang et al., 2013).

Non-microbial CH_4 release has been shown to occur under ultraviolet (UV) irradiation and elevated temperatures from dry and detached fresh plant material (McLeod et al., 2008;Vigano et al., 2008;Bruhn et al., 2009). Pectin has been considered a source of CH_4 production primarily because of its high degree of methylation (Keppler et al., 2008;Bruhn et al., 2009;Messenger et al., 2009). A reaction of reactive oxygen species (ROS) with pectic polysaccharides was suggested as a possible route to CH_4 formation under UV radiation (Messenger et al., 2009). From plant matter CH_4 can be derived from other structural components as shown with commercially purified lignin and cellulose (Vigano et al., 2008), leaf surface waxes (Bruhn et al., 2014), and even from ascorbic acid under highly oxidative conditions (Althoff et al., 2010). For further information on CH_4 formation, from dry and fresh detached plant matter and specific plant structural compounds, the reader should refer to the recent reviews of Bruhn *et al.* (2012) and Wang et al. (2013).

Stable isotope analysis has been employed to demonstrate that living plants release CH_4 at rates ranging from 0.16 to 12 ng g^{-1} dry weight (DW) h^{-1} (Brüggemann et al., 2009;Wishkerman et al., 2011). Isotope labelling studies involving young poplars grown in a $^{13}CO_2$ atmosphere (Brüggemann et al., 2009) together with several plants cell culture experiments with ^{13}C-labelled sucrose (Wishkerman et al., 2011) confirmed that CH_4 is produced in plants per se and not by their associated micro-organisms. Here it was suggested that CH_4 formation was the result of abiotic stress factors to which the plant had been exposed, an idea supported by findings of two other recent studies (Qaderi and Reid, 2009, 2011). Although these results would support the contention that living plants release CH_4, the mechanism of its formation and its precursors still remain unknown.

A first step towards delineating the formation pathway was recently undertaken by Wishkerman et al. (2011) who investigated the influence of the toxin sodium azide (NaN_3) on cell cultures such as tobacco, sugar beet and grape vine. As sodium azide is known to disrupt electron transport flow at the cytochrome c oxidase (complex IV) in plant mitochondria, findings of the study suggested that disturbance of the electron transport chain (ETC) cause CH_4 formation in plant cells. Thus CH_4 formation in living plants may be an integral part of cellular responses to changes in oxidative status present in all eukaryotes. It has been hypothesized that compounds such as phosphatidylcholine or methionine (Met) might be carbon precursors of CH_4 in both animal and plant cells particularly when under stress conditions (Wishkerman et al., 2011;Bruhn et al., 2012)

Furthermore, very recently a chemical reaction showing that CH_4 can be readily formed from organosulfur compounds under oxidative conditions, ambient atmospheric pressure and temperature has been suggested (Althoff et al., 2014). In the first step of the reaction, methyl sulfides are oxidised to the corresponding sulfoxides. Then, in the next step, demethylation of the sulfoxide via homolytic bond cleavage leads to methyl radical formation and finally to CH_4. In the same study tobacco plants (*Nicotiana tabacum*) grown under sterile conditions were supplemented with positionally labelled Met, where only the methyl group (–S–CH_3) was enriched with ^{13}C atoms. These experiments provided the first evidence that the thio-methyl group of Met is a parent compound of CH_4 in tobacco plants. The potential role of methionine and its oxidation product methionine sulfoxide (MSO) in CH_4 biosynthesis in living plants was also suggested by Bruhn *et al.* (2012). Additionally methionine has been shown to be involved in CH_4 formation in saprotrophic fungi (Lenhart et al., 2012).

In this study we investigated methionine, an amino acid that plays a major role in trans methylation reactions, as a precursor of CH_4 biosynthesis in living plants under dark conditions. We employed *Lavandula angustifolia* which was previously shown to produce significant amounts of CH_4 both under normal and stress conditions such as physical wounding and anoxia (Wang et al., 2009;Wang et al., 2011b;Wang et al., 2011a). We made use of stable isotope techniques and ^{13}C-positionally labelled methionine to verify in vivo formation and identify the carbon atom precursor of CH_4.

MATERIAL & METHODS

Cultivation of plants

Commercially available *L. angustifolia* plants grown to approximately 20-25 cm were purchased for the initial experiment (consecutive treatment experiment). Plants used in the second experiment (parallel treatment experiment) were grown in a greenhouse until a height of 20 cm was reached. Trace gas measurements were carried out at 22 ± 2 °C and 100 % humidity under dark conditions.

Supplementation of *L. angustifolia* with ^{13}C-methionine

L. angustifolia plants were removed from pots and the soil washed from the roots. For incubation, each plant was placed inside a 2.5 l flask (Weck©, Hanau, Germany) containing 50 ml distilled H_2O and then sealed with a lid held in place with a rubber band. To facilitate headspace sampling each lid had a central hole fitted with a silicone stopper. In the ^{13}C-Met labelled flasks the distilled water was supplemented with 0.4 mM ^{13}C-Met. We used a positionally labelled Met where only the methyl group (–S–CH_3) was enriched with ^{13}C atoms. After sealing, measurements of headspace CH_4 concentrations and ^{13}C-CH_4 signatures were conducted over a four day period. Different incubation periods and measurement intervals were necessary to correspond with the available schedule times for use of the isotope measurement system. For isotope measurements the incubation flask was directly connected to the IRMS system.

Physical stressing of plants

Detached leaves of *L. angustifolia* have previously been shown to release significant amounts of CH_4 both under normal and stress conditions, such as physical wounding by cutting (Wang et al., 2011b). However, stressing of plants by cutting is a time-consuming process and a rapid, stress-induced increase in CH_4 emissions might occur during this process and before the flasks were sealed. Therefore, physical stress in our study was performed by gently compressing individual leaves of each plant between fingertips for a short time period (one to two seconds). This procedure took approximately one minute for each plant, i.e. until the majority of the leaf surface area was injured.

Analytical procedures

Gas samples were analysed within 24 h on a gas chromatograph (Shimadzu GC-14B, Kyoto, Japan) with a flame ionization detector (CH_4) and an electron capture detector (N_2O, CO_2) operated at 230 and 320 °C with N_2 as carrier gas (25 ml min^{-1}) (Kammann et al., 2009;Kammann et al., 2012). The GC column (PorapakQ, Fa. Millipore, Schwallbach, mesh 80/100) was 3.2 m long and 1/8 inch in diameter. The length of the precolumn was 0.8 m. The GC gas flow scheme and automated sampling was that of Mosier and Mack (1980) and Loftfield et al. (1997), and peak area integration was undertaken with the Software PeakSimple, version 2.66. The s.d. of the mean of six atmospheric air standard samples was below 1.0%, 0.5%, and 0.2% for CO_2, N_2O, and CH_4, respectively.

Continuous flow isotope ratio mass spectrometry (IRMS) for measurement of $\delta^{13}C$ values of CH_4

Headspace gas from the flasks was transferred to an evacuated sample loop (40 mL). Interfering compounds were separated by GC and CH_4 trapped on Hayesep D. The sample was then transferred to the IRMS system (ThermoFinnigan Delta^{plus} XL, Thermo Finnigan, Bremen, Germany) via an open split. The reference gas was carbon dioxide of high purity (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) with a known $\delta^{13}C$ value of -23.64 ‰ (V-PDB). All $\delta^{13}C$ values were normalized relative to V-PDB (Vienna Pee Dee Belemnite) using a CH_4 standard. Samples were routinely analysed three times (n = 3) and the average standard deviations of the GC/C/IRMS measurements were in the range of 0.1 to 0.3 ‰.

All $^{13}C/^{12}C$ -isotope ratios are expressed in the conventional δ notation in per mil versus V-PDB, [‰] using the following equation (Eq. 1):

$$\delta^{13}C_{V\text{-}PDB} = (({}^{13}C/{}^{12}C)_{sample} / ({}^{13}C/{}^{12}C)_{standard}) - 1. \quad \text{(Eq. 1)}$$

Statistics

For the evaluation of results from experiments with *L. angustifolia* plants we utilised the software package SPSS Version 20. In the initial experiment a paired sample *t-test* was used to compare the rates of CH_4 production between consecutive treatments “non-stressed” and “stressed” on the same plants (Levene-Test was 0.394 after log transformation of data). For the purposes of brevity from this point forward this experiment will be referred to as the consecutive treatment experiment. The second experiment where simultaneously different samples were used to compare between

149 "non-stressed" and "stressed" treatments is referred to as the parallel treatment
150 experiment. The CH_4 and $\delta^{13}CH_4$ emission ratio between stressed and non-stressed
151 plants (Tab. 1) was calculated by using the mean value of the samples 9-11 as reference
152 for the non-stressed plants.
153 Data from experiment 2 and differences between experiments 1 and 2 were analysed by
154 an independent samples t-test. Differences between results are given as significant ($p < 0.05$) or highly significant ($p < 0.001$).
156 In the case where no significant differences occurred between the control (no stress
157 applied or Met added) and the treatment where only Met was added, CH_4 emission rates
158 and CH_4:CO_2 ratios were pooled in order to calculate the mean values.
159

160 **RESULTS**

161 ***L. angustifolia* supplemented with –S–CH_3 positionally labelled Met and the effect**
162 **of physical stress**

163 We investigated CH_4 emissions of *L. angustifolia* supplemented with ^{13}C-positionally
164 labelled Met ($^{13}CH_3$-Met; only the methyl group –S–CH_3 was enriched with ^{13}C atoms).
165 under stressed and non-stressed conditions. The incubations were carried out in total
166 darkness in order to exclude possible light effects and to measure the gross respiration
167 of plants. The effect of physical stress was investigated in parallel experiments where in
168 each case three different individual plants were placed under "stressed" and "non-
169 stressed" conditions. Additionally, an experiment was performed where CH_4 emissions
170 were first measured on intact plants, and then after physical stress was applied to these
171 plants, CH_4 emissions were again measured.

The CH_4 emission rates of non-stressed *L. angustifolia* plants ranged from 0.06 to 0.57 ng CH_4 g^{-1} DW h^{-1}, with a mean value of 0.33 ±0.16 ng CH_4 g^{-1} DW h^{-1} (Table 1). Physical stress enhanced CH_4 emissions by a factor of 5.7 up to a mean CH_4 emission rate of 1.7 ±0.57 ng g^{-1} h^{-1} (n = 8). The effect of physical stress was significant both for plants undergoing consecutive treatment (paired t-test with *p = 0.002,* n = 5) and those in the parallel treatment experiment (independent samples t-test with p = 0.005). Addition of Met did not affect the CH_4 production of non-stressed plants (Tab. 1, comparison of flasks 6-8 vs. 9-11; *p = 0.632*, n = 3).

L. angustifolia (n = 2) supplemented with $^{13}CH_3$-Met showed a strong response in the isotope signal with $\delta^{13}C(CH_4)$. After addition of $^{13}CH_3$-Met to the roots (n = 2) $\delta^{13}C(CH_4)$ values increased from -47 at time 0 to 29 and 97 ‰ after 20 h in non-stressed (Fig. 1d) and stressed plants (Fig. 1f), respectively, whereas in the control samples (n = 3) only a marginal change (± 1 ‰) was noted (Fig. 1b). Mean changes in $\Delta^{13}CH_4$ signature were 0.39 ±0.16 ‰ g^{-1} h^{-1} for the non-stressed plants. After stressing, a strong response in the mixing ratio of CH_4 occurred (Fig. 1), reaching a $\Delta^{13}CH_4$ up to 0.64 ±0.30 ‰ g^{-1} h^{-1} (Tab. 1). The ^{13}C signature increase in headspace-CH_4 over time (Fig. 1d, f) clearly shows that the methyl group of Met is a precursor of CH_4 in plants under both non-stressed and stressed conditions.

For non-stressed and stressed plants, 0.008 and 0.009 % of $^{13}CH_3$-Met molecules were on average daily converted to CH_4 assuming that all $^{13}CH_3$-Met was taken up by the plant.

196 The ratio of CH_4 emissions (5.7) and $\Delta^{13}CH_4$ h^{-1} g^{-1} (1.9) between stressed and non-
197 stressed plants (Tab. 1) indicated a higher effect of physical stress on CH_4 formation
198 than on the changes in $\delta^{13}C(CH_4)$ values.
199
200 **Respiration**
201 During incubation of *L. angustifolia*, changes in the CO_2 mixing ratio were also
202 monitored. Since the incubation was conducted in the dark no CO_2 uptake took place and
203 thus changes in the mixing ratio only reflect the plants respiration. Respiration was on
204 average for non-stressed and stressed plants 0.36.±0.14 and 0.50 ±0.16 mg CO_2 g^{-1} h^{-1},
205 respectively.

206 The CO_2 production by respiration reflects metabolic activity of the plants, and to relate
207 CH_4 production to metabolic activity we related CH_4 formation to respiration by
208 calculating the CH_4:CO_2 ratio [mol:mol x 10^6] separately for each sample. Figure 2 shows
209 the CH_4 and CO_2 production rates and the respective CH_4:CO_2-ratio, which was 2.0 ±1.1
210 for the non-stressed plants (n = 6) and 8.5 ± 1.8 for the plants where physical stress was
211 applied (n = 3). Thus, physical damage resulted in a 4-fold increase in the CH_4:CO_2 ratio
212 (*p = 0.004*). Supplementation of plants with Met did not affect the CH_4 or CO_2 emission
213 rates (p > 0.1).
214
215

DISCUSSION

Effects of physical stress on CH_4 production

Methane emission rates of 0.33 ±0.16 and 1.71 ±0.57 ng g^{-1} DW h^{-1} were observed for *L. angustifolia* under non-stressed and physically stressed conditions, respectively. The upper range (under physical stress) here is still considerably lower (one to two orders of magnitude) than the range of CH_4 emission rates from intact living plants (12 to 370 ng g^{-1} DW h^{-1}) reported by Keppler et al. (2006) and those reported from crop species under environmental stress (Qaderi & Reid, 2009; Qaderi & Reid, 2011).

Without the application of physical stress the calculated emission rates were considerably lower (factor of three) with maximum values reaching 0.57 ng CH_4 g^{-1} DW h^{-1} and are similar to those reported in previous studies employing stable carbon isotope labelling tools (Bruggemann *et al.*, 2009; Wishkerman *et al.*, 2011). The very low values reported here again highlight the benefit of using stable isotope tools as it would be very difficult to clearly monitor aerobic CH_4 formation in non-stressed living plants without their employment. This might also include the sensitive optical online methods employed in several studies (Dueck *et al.*, 2007; Beerling *et al.*, 2008). However, these spectroscopic methods might be able to measure CH_4 formation in plants under particular stress conditions.

Earlier studies (Wang *et al.*, 2009; Wang *et al.*, 2011a; Wang *et al.*, 2011b) already revealed a significant increase in CH_4 emissions after physical injury and suggested reactive oxygen species (ROS) to be involved in the cleavage of methyl groups from pectin and/or lignin (Wang et al., 2011b; Keppler et al., 2008; McLeod et al., 2008; Messenger et al., 2009). Our finding of close to a 4-fold increase in CH_4 emissions after

application of physical stress is within the range of the values reported by Wang et al. (2011b) where the CH_4 emission rates of *L. angustifolia* were 0.42 ±0.05 and 6.32 ±0.69 ng g^{-1} h^{-1} for intact leaves and after wounding, respectively. In that study observations of CH_4 emission rates of intact plants (0.4 ng g^{-1} h^{-1}) and after wounding (0.7 ng g^{-1} h^{-1}) for 56 plant species were reported. The discrepancy in the physical stress-induced response in CH_4 emissions of *L. angustifolia* might be due to an exceptionally high response of this species when compared to other species tested by Wang et al. (2011b). Differences in the stress-response between samples (ratio between stressed and non-stressed plants was in a range of 3.3 to 11.2) in this study might be due to a different degree of injury during the compression of the individual leaves of each plant.

CH_4:CO_2-ratio

Based on the CH_4 and CO_2 emission rates the calculated CH_4:CO_2 ratio [mol:mol x 10^6] of intact plants was 2.0 ±1.1 (n = 6), i.e. per mol CO_2 2.0 µmol CH_4 was released by the plants.

This is close to the ratios observed for saprotrophic fungi including *Pleurotus sapidus, Stereum sanguinolentum, Panaerochaete chrysosporium* where the CH_4:CO_2-ratio was 3.5 ±1.92 mol:mol x 10^6 or 1.15 ±0.70 ng:mg (Lenhart et al., 2012) and cryptogams including, mosses and , lichens (n =31) with a ratio of 2.58 ±2.60 mol:mol x 10^6 or 0.94 ±0.95 ng:mg (Lenhart et al., submitted). The CH_4:CO_2-ratio of *L. angustifolia* tends to be lower, but, as indicated by the standard deviation, this difference is probably not significant.

The physical stress-induced increase in the CH_4:CO_2 ratio (factor 4) of up to 8.5 ±1.8 was caused by a significant increase in CH_4 emissions accompanied only by a small increase in respiration. The overall stress-induced increase in respiration of *L. angustifolia* compared to non-stressed plants was 40 % (n.s., $p > 0.3$), which is similar to that observed by Wang et al. (2009) who reported a 50 % increase in CO_2 emissions for plants physically wounded by cutting compared to those left uncut. The CH_4:CO_2-ratio might be a useful tool to relate CH_4 emissions to metabolic activity in the dark and to compare CH_4 emission rates of different species obtained under different conditions.

Methionine as a precursor of CH_4 in plants

Based on the addition of $^{13}CH_3$-Met and stable carbon isotope measurements it was possible to clearly monitor $^{13}CH_4$ formation in *L. angustifolia*. The increase in the $\delta^{13}CH_4$ signature of CH_4 released by the plants after supplementation with $^{13}CH_3$-Met (Fig. 1, Tab. 1) is a strong indicator that the methyl thiol group of Met is a CH_4 precursor during metabolic processes. When plants were physically stressed, there was not only an increase in CH_4 release but also an increase in its $^{13}CH_4$ signature (Fig. 1, Tab. 1).

It is important to note that our data cannot be used to conclude that the methionine *per se* is a direct precursor of CH_4 in plants. Indeed, since most methionine resides in proteins it is much more likely to be the source of CH_4 production after oxidation by ROS as suggested by Bruhn *et al.* (2012). These workers based their hypothesis on the finding that dimethyl sulfoxide (DMSO) could act as a hydroxyl radical scavenger in cells and produces CH_4 via a radical mechanism (Repine *et al.*, 1981). They argued that when

283 the amino acid methionine is oxidized the first oxidized product is methionine sulfoxide,
284 where the side chain is similar to DMSO. Therefore methionine sulfoxide might also be a
285 precursor of CH_4 biosynthesis. The concentration of the free amino acid in normal plant
286 tissues is <100 µM (Inaba *et al.*, 1994), while that found in protein is two to three orders
287 of magnitude higher (Bruhn *et al.*, 2012). Methionine residues are known to be prone to
288 oxidation by H_2O_2 (Levine *et al.*, 1996; Moller *et al.*, 2007) to form methionine sulfoxide.
289 Based on the calculations of Bruhn *et al.* (2012) it would only require a conversion rate
290 and release of less than one CH_4 molecule per hour for every 1000 protein methionine
291 sulfoxide to explain a release rate of 10 ng CH_4 g^{-1} DW h^{-1} by intact plant tissues. These
292 considerations are strongly supported by findings reported in a recent chemical study
293 (Althoff et al. 2014) which suggest that under oxidative conditions demethylation of
294 sulfoxides might lead to CH_4. In that study methyl groups of organosulfur compounds
295 were shown to be efficiently converted to CH_4 when using iron(II/III), hydrogen
296 peroxide and ascorbic acid as reagents. It is thought that methyl sulfides are first
297 oxidised to the corresponding sulfoxides and demethylation of the sulfoxide via
298 homolytic bond cleavage leads to methyl radical formation and then to CH_4.

299 However, in our study we focused on the identification of the precursor compound of
300 CH_4 in living plants and the location of the carbon atom within the precursor molecule.
301 Thus additional investigations and measurements concerning the formation of ROS,
302 distinguishing between free methionine and that in protein, as well as the possibility of
303 methionine residue oxidation to Met-SO will need to be undertaken.

304 Next to methionine, several other organic compounds with methyl- or methoxyl groups
305 were already identified to serve as a substrate for CH_4 formation in plants (McLeod et al.,

306 2008;Vigano et al., 2008;Bruhn et al., 2009, Keppler et al., 2008, Messenger et al., 2009).
307 Thus, in our experiment besides Met also other organic compounds such as pectin, lignin
308 or choline might serve as further methyl-group donors for CH_4 formation. A detailed
309 discussion on potential plant precursors of CH_4 was recently provided by Wang et al.
310 (2013).

311

312 **Conclusions and Outlook**

313 Our study is an important step in deciphering the precursor compounds involved in CH_4
314 biosynthesis in living plants. The employment of $^{13}CH_3$-Met clearly showed that the
315 sulphur bonded carbon atom of the methyl group in methionine was transferred to CH_4
316 in *L. angustifolia*. Knowledge of the precursor compound and identification of the carbon
317 atom involved is a first step towards elucidating the pathway of its formation. An
318 understanding of the mechanisms of CH_4 formation in living plants may reveal if the
319 process has a more general physiological role. Further experiments where other stress
320 factors in combination with $^{13}CH_3$-methionine are investigated should provide further
321 insight into the pathways involved in CH_4 formation.

322 Although it has been argued that plants may not produce CH_4 as an end-product or by-
323 product of their metabolism (Nisbet et al., 2009), our results would strongly indicate
324 that plants do contain a mechanism for CH_4 production. The stress-induced increase in
325 CH_4 emissions together with studies on sterile plants (Wishkerman *et al.*, 2011) or
326 cryptogamic covers supplemented with diverse inhibitors (Lenhart *et al.*, submitted), do
327 not support the hypothesis of a noteworthy contribution of *Archaea* to plant-derived

328 CH_4-formation in this study. However, we did not scan for methanogenic Archaea in the
329 wooden parts of lavendula plants to exclude Archaeal-derived CH_4 formation as
330 postulated by Covey et al. (2012). Furthermore, it is considered that organosulfur
331 compounds might play an important role in the formation of CH_4 in living plants
332 particularly when under stress conditions.

333

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339

Figure legends

Figure 1: Mixing ratios (a, c, e) and $\delta^{13}C$ values (b, d, f) of CH_4 produced by *L. angustifolia* (n = 3) in the control flasks (a, b) without addition of methionine or physical stress, supplemented with ^{13}C-labelled Met without physical stress (c, d), and supplemented with ^{13}C-labelled Met and the application of physical stress (e, f). Different symbols mark the replicates per treatment.

Figure 2: (a) Methane and CO_2 emissions of *L. angustifolia* supplemented with ^{13}C-labelled Met under non-stressed conditions and after application of physical stress, and (b) CH_4 to CO_2 emission ratio. Control plants were not supplemented with Met or exposed to physical stress.

353 **Table 1:** Rates of formation and $\delta^{13}C$ signature increase of CH_4 released during
354 incubation of *L. angustifolia* plants before and after physical stress. Samples 1-5 were
355 measured in experiment 1 (consecutive treatment experiment, measurements at day 1,
356 non-stressed and 2, stressed), samples 6-14 in experiment 2 (parallel treatment
357 experiment). The difference in the CH_4 rate increase between the treatments "non-
358 stressed" and "stressed" was significant (*p = 0.003 and p = 0.005 for Exp. 1 and Exp. 2,*
359 *respectively*). *supplemented with ^{13}C-Met

Sample	CH_4 [ng g^{-1} h^{-1}]			$\Delta^{13}CH_4$ [‰ g^{-1} h^{-1}]		
	non-stressed	stressed	Ratio stressed/non-stressed	non-stressed	stressed	Ratio stressed/non-stressed
1	0.52	1.70	3.3	-	-	
2	0.57	1.87	3.3	-	-	
3	0.43	2.41	5.6	-	-	
4*	0.31	1.21	3.9	0.27	0.39	1.4
5*	0.18	1.67	9.1	0.11	0.34	3.2
6	0.37	-		-	-	
7	0.39	-		-	-	
8	0.14	-		-	-	
9*	0.24	-		0.36	-	
10*	0.06	-		0.43	-	
11*	0.40	-		0.79	-	
12*	-	1.26	5.4	-	0.78	1.5
13*	-	0.98	4.2	-	0.63	1.2
14*	-	2.61	11.2	-	1.07	2.0
Mean ±sd	**0.33 ±0.16**	**1.71 ±0.57**	**5.7 ±2.9**	**0.39 ±0.25**	**0.64 ±0.30**	**1.86 ±0.81**

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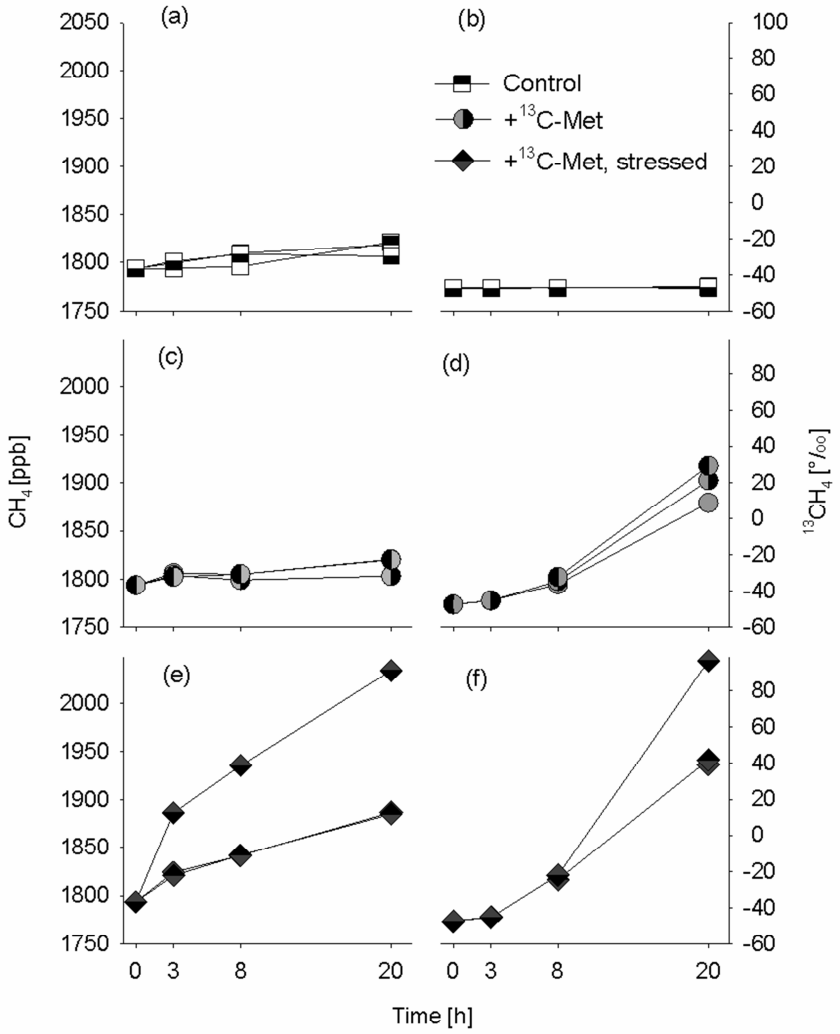

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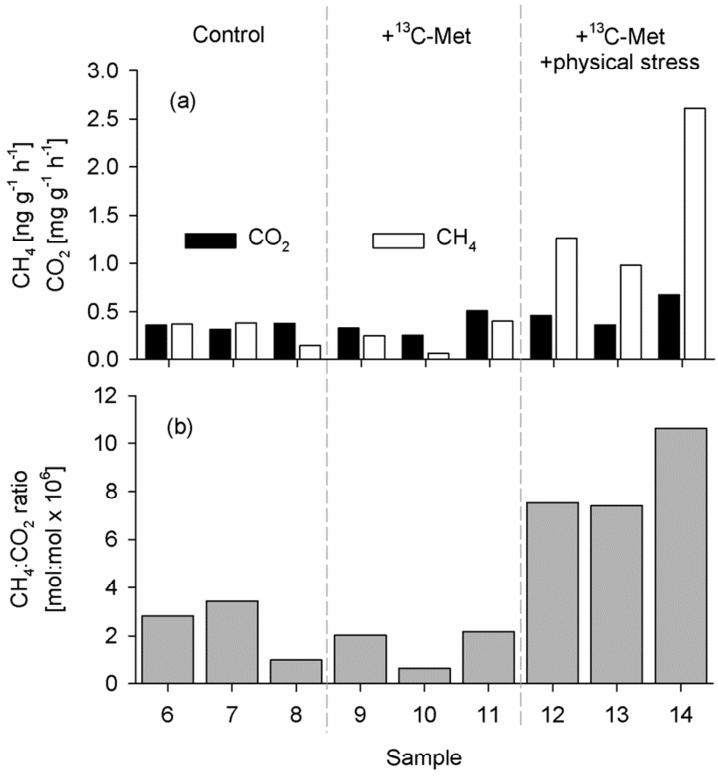
464 Figure 1



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466 Figure2