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6 A stable isotope titration method to determine the contribution of acetate 7 disproportionation and carbon dioxide reduction to methanogenesis

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5
6 *Abstract*

7 A novel stable isotope titration approach was developed to determine the
8 contributions to total methane production made by CO_2 reduction and the disproportionation
9 of acetate in anoxic environments. $^{13}CH_4$, $^{12}CH_4$, $^{13}CO_2$ and $^{12}CO_2$ production rates were
10 measured in the headspace of replicate anaerobic microcosms titrated with increasing
11 amounts of ^{13}C labelled substrates. The contribution of CO_2 reduction was calculated from the
12 linear relationship between ratios of labelled and total CH_4 production and ratios of labelled
13 and total CO_2 after the addition of ^{13}C-bicarbonate. In the case of acetoclastic methanogenesis
14 rates of $^{13}CH_4$ and $^{12}CH_4$ production were fitted to a model based on an assumption that the
15 relationship between the concentration of ^{13}C-labelled acetate and the rates of labelled and
16 unlabelled methane production followed Michaelis-Menten kinetics. A comparison of the raw
17 data with the model supported the assumption and provided both an estimate of the
18 contribution of acetate to methane production and an estimate of the size of the indigenous
19 acetate pool without the need to measure acetate directly. The method was applied to a
20 freshwater sediment in the English Lake District where it was found that 66.3 (se 4.9) % of
21 methane production was due to acetate disproportionation and 28.9 (se 1.9) % of methane
22 production resulted from CO_2 reduction. This is in agreement with theoretical predictions and
23 other empirical measurements of methanogenesis.

1 *Introduction*

2 In sedimentary environments methanogenic archaea produce methane primarily by
3 reduction of CO_2 by hydrogen (Hydrogenotrophic methanogenesis), disproportionation of
4 acetate (acetoclastic methanogenesis), and to a lesser extent from a range of methylated
5 substrates e.g. methylamines. The relative contributions of these processes vary between
6 different environments and, although not fully explained, appear to be dependent on the
7 extent to which anaerobic degradative processes other than methanogenesis, (e.g.
8 fermentation, acetogenesis, sulfate, iron and nitrate reduction) affect the availability of H_2 ,
9 acetate and other methanogenic precursors (Conrad 1999). The use of different substrates by
10 CO_2 reducing and acetoclastic methanogens is likely to affect their competitive and
11 syntrophic interactions, therefore, to fully understand the biologically mediated geochemical
12 processes occurring in anaerobic environments it is desirable to have separate estimates of at
13 least the two major pathways that contribute to methane production.

14 Direct measurement of these processes separately is typically achieved by radiometric
15 measurement of $^{14}CH_4$ production in anaerobic microcosms after the addition of ^{14}C labelled
16 acetate or bicarbonate. (e.g. Winfrey and Zeikus 1979, Nusslein *et al.* 2001, Bonch-
17 Osmolovsskaya *et al.* 2003). Aside from safety aspects and the expense of using
18 radioisotopes, this approach also requires an accurate knowledge of the steady state
19 concentrations of both unlabelled bicarbonate and unlabelled acetate in order to accurately
20 determine the specific activity of the substrate pools (de Graaf *et al.* 1996). Because added
21 bicarbonate rapidly equilibrates with dissolved and gaseous CO_2 the contribution of H_2/CO_2
22 can be estimated by the measurement of the specific radioactivity of CH_4 and CO_2 in head
23 space gas samples (Conrad *et al.* 1989). However, in addition to head space gas analysis to
24 determine the contribution of acetoclastic methanogenesis, unlabelled acetate concentrations

1 must be measured separately on extracts of the sediment pore water. Here we report a novel
2 technique using stable isotope labelled substrates where the initial ratio of $^{13}C/^{12}C$ in the
3 acetate and bicarbonate pools was titrated by adding increasing amounts of labelled substrate
4 followed by measurement of labelled and unlabelled CH_4 and CO_2 in head space gases by
5 GC-MS. The stable isotope approach does not require the concentration of acetate in pore
6 water to be determined to derive an estimate of the contribution of acetoclastic
7 methanogenesis to methane production. In addition because of the relative cost, safety and
8 simplicity of the method (i.e. measurement of non-radioactive head space gasses by GCMS),
9 a large number of replicate experiments can be run simultaneously, increasing the precision
10 of the analysis. It should be noted that this technique is different to the indirect method of
11 estimating methanogenic pathways by the GC-C-IRMS measurement of the natural
12 abundance of ^{13}C in gas and liquid samples (Conrad *et al.* 2002).
13
14 *Methods*
15 The method for determining the contribution of acetoclastic methanogenesis is
16 analogous to a method developed to estimate substrate uptake in aquatic ecosystems (Wright
17 and Hobbie 1966). It is based on the assumption that in a well mixed aqueous environment
18 the uptake of a substrate by an individual microbial cell follows Michaelis-Menten kinetics
19 (Wetzel 1983). Accordingly when sediments are titrated with different concentrations of ^{13}C-
20 labelled acetate, the ratio of labelled to unlabelled acetate in the sediment will change and the
21 relationship between initial ^{13}C-acetate concentrations and labelled CH_4 production should
22 follow a rectangular hyperbola. Assuming, in the short term (i.e. minutes to hours), that no
23 stimulation of the overall rate of methane production occurs through increases in substrate
24 concentration (see below), labelled methane production should be first order with respect to

1 substrate added at very low concentrations. At high concentrations of labelled substrate i.e.
2 where the majority of substrate pool is labelled, labelled methane production should be zero
3 order (saturated) with respect to substrate. Accordingly the rate of production of $^{13}CH_4$ should
4 increase with the amount of ^{13}C substrate according to equation 1. Here *B* is the production
5 rate of $^{13}CH_4$ when labelled methane production is zero order (saturated) with respect to
6 labelled substrate concentration and *K* represents the labelled substrate concentration when
7 the production rate of labelled methane is ½*B*.

8
9

10 $$^{13}\mathrm{CH}_4 = \frac{B[\mathrm{S}_{\mathrm{labelled}}]}{K + [\mathrm{S}_{\mathrm{labelled}}]} \qquad \text{Eq. 1}$$

11

12 The rate of production of unlabelled methane, ($^{12}CH_4$) can also be measured, which when
13 combined with the $^{13}CH_4$ data gives the total production of methane. *A priori* we expect the
14 total production of methane to be unaffected by the concentration of labelled substrate (This
15 is a critical assumption of the model and is discussed below). Consequently, we expect the
16 production of $^{12}CH_4$ to be governed by equation 2 where *A* is the total production rate of
17 methane. In this equation the production rate of $^{12}CH_4$ decreases as the concentration of ^{13}C-
18 labelled substrate increases.

19

20 $$^{12}\mathrm{CH}_4 = A - \frac{B[\mathrm{S}_{\mathrm{labelled}}]}{K + [\mathrm{S}_{\mathrm{labelled}}]} \qquad \text{Eq. 2}$$

21

22 To test the relationship between initial ^{13}C-substrate concentrations and
23 labelled/unlabelled CH_4 production, grab samples of surface sediment were obtained from a
24 wetland area on the margins of Rydal Water, Cumbria, UK (54°21′N, 2°51′W). Sediment

1 microcosms were prepared from bulk sediment slurries (2.5 cm^3 sediment with 2.5 cm^3
2 overlying water) in sterile serum bottles (14 cm^3 capacity, Sigma-Aldrich, UK). Sediment
3 microcosms were sealed with crimp top butyl rubber stoppers after repeated flushing with
4 oxygen-free nitrogen. The microcosms were amended with sodium acetate [2-^{13}C] or Na
5 $H^{13}CO_3$ (50 mM degassed stock solutions) to give a range of initial concentrations of labelled
6 substrate (0 mM, 0.005 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5mM). Triplicate microcosms
7 were prepared at each concentration, and were incubated on a rotary shaker (150 rpm).
8 Headspace samples (100 μl) were taken at time zero and thereafter at 1 h intervals, using a
9 helium flushed push lock gas-tight syringe (SGE, Australia). Labelled and unlabelled CO_2
10 and CH_4 in headspace gas were analyzed by single ion monitoring ($^{13}CO_2$ (m/z =45), $^{12}CO_2$
11 (m/z =44), $^{13}CH_4$ (m/z = 17), $^{12}CH_4$ (m/z =15)) using a gas chromatograph mass spectrometer
12 (Trio 1000 MS; Fisons, UK) fitted with a column packed with Pora Plot Q stationary phase
13 (25m x 0.25 mm i.d.; Chrompack, NL) and helium as the carrier gas. The injection (250 ^{o}C)
14 and oven (35°C) temperature were constant throughout. Gas sample injections onto the
15 column were made at 1 minute intervals to allow for the rapid processing of samples. Peak
16 areas were calibrated using standardized gas mixtures (CO_2 and CH_4) in air (0, 1, 0.1, 0.05,
17 0.025%). Due to the interference of the primary and secondary ions produced by the
18 fragmentation of the labelled and unlabelled CH_4, $^{13}CH_4$ was measured as the primary ion
19 $^{(13}CH_4^+$, m/z = 17) while $^{12}CH_4$ was measured as its secondary ion ($^{12}CH_3^+$ m/z = 15). The
20 tertiary ion of $^{13}CH_4$ ($^{13}CH_2^+$, m/z = 15) produces an ion count that is 14% of the primary ion
21 so it was necessary to make a correction for this in the measurement of $^{12}CH_4$ after
22 calculation of the $^{13}CH_4$ concentration..
23 In sediment microcosms amended with ^{13}C-acetate or ^{13}C-bicarbonate, rates of
24 labelled and unlabelled methane production were calculated from their initial linear

1 accumulation. The linear accumulation of methane occurred in the first five hours after
2 addition of labelled acetate . This short term gas sampling strategy was adopted to measure
3 production rates at the initial added substrate concentration and, in the case of acetate, before
4 any longer term stimulation of overall methane production might occur. For the acetate
5 experiments estimates for the parameters governing the Michaelis-Menten kinetics, (*B* and *K*
6 and of the total production of methane, *A*), were obtained by fitting a statistical model based
7 on equations 1 and 2 to the ^{13}C and ^{12}C data sets using non-linear regression methods. The
8 model fitted is as follows:
9

10 $^{13}CH_4$ production: $$y_{ij}^{13} = \frac{Bs_i}{K + s_i} + \varepsilon_{ij}^{13}$$ Eq. 3.

11 $^{12}CH_4$ production: $$y_{ij}^{12} = A - \frac{Bs_i}{K + s_i} + \varepsilon_{ij}^{12}$$ Eq. 4.

12
13
14 Where s_i denotes the *i*th substrate concentration employed in the experiment
15 (*i*=1,2,3,4,5 corresponding to the five concentrations of ^{13}C-acetate used). $y_{i1}^{12}, y_{i2}^{12}, y_{i3}^{12}$ and
16 $y_{i1}^{13}, y_{i2}^{13}, y_{i3}^{13}$ are respectively, the rates of production of $^{12}CH_4$ and $^{13}CH_4$ observed in three
17 replicate microcosms at concentration i. ε_{ij}^{12} and ε_{ij}^{13} are error terms. In the model the residual
18 terms are assumed to have a Normal distribution. For the $^{13}CH_4$ observations the residual
19 standard deviation, σ_{13}, is not assumed to be equal to the corresponding standard deviation,
20 σ_{12}, for the $^{12}CH_4$ observations. We chose a model with different error terms based on a
21 preliminary examination of the data which indicated that the $^{12}CH_4$ values were inherently
22 more variable than the $^{13}CH_4$ values obtained. The model was fitted by maximum likelihood
23 (ML) and the standard errors of the parameter estimates were obtained from the expected
24 information matrix. The data analysis is not trivial and we have written a computer program

1 (MMlink) which calculates *B* and *K* and total production of methane, *A*, from methane
2 production rates. In addition the program carries out likelihhod ratio tests to validate the
3 assumptions made (see below). The program can be downloaded from
4 http://www.mas.ncl.ac.uk/~njnsm/research/mmlink/intro.htm. In addition to the software the
5 web site gives details of the technical statistical issues behind the method and allows the user
6 to check the installation of the program using the methane production data generated in this
7 study. The software for the maximum likelihood analysis was written in FORTRAN and uses
8 the simplex algorithm (Nelder and Mead 1965).
9
10 *Results and discussion*
11 The rate of methanogenesis measured in Rydal Water sediments was 0.229 (se
12 0.0113) µmol h^{-1} cm^{-3}. For acetoclastic methane production, estimates of *A, B* and *K* derived
13 from the likelihood analysis are provided in Table 1. The residuals of the ^{13}C and ^{12}C data
14 were assumed to be normally distributed and the validity of this assumption was confirmed
15 using probability plots (for details see
16 http://www.mas.ncl.ac.uk/~njnsm/research/mmlink/intro.htm). A key assumption of the
17 model was that within the time frame of the incubation, the addition of acetate did not
18 stimulate overall methane production rates. From the fitted model, total methane production
19 (*A*) was found to have a relatively small error over the range of acetate concentrations tested
20 (0.229 (se 0.011) µmol h^{-1} cm^{-3}). Furthermore a graphical assessment of the fitted curves
21 (Fig. 1.) strongly indicated that increases in ^{13}C methane production related to additions of
22 labelled acetate were mirrored by equivalent decreases in ^{12}C methane production rates and
23 the overall rate of methane production was constant.

1 A further assessment of the assumption that the total rate of methane production was
2 unaffected by substrate concentration was made by fitting an extension of the model
3 described by equations 3 and 4. Equation 3 is unchanged in the extended model whereas
4 equation 4 is replaced by:

5 $^{12}CH_4$ production: $$y_{ij}^{12} = A + Cs_i - \frac{Bs_i}{K + s_i} + \varepsilon_{ij}^{12}$$ Eq. 4a.

6 Here Cs_i represents the contribution of added labelled acetate, S_i, to the total rate of methane
7 production. An assessment of whether the total rate of methanogenesis is increased by
8 labelled substrate addition was made by testing the hypothesis that $C = 0$. Using a likelihood
9 ratio test (a statistical assessment of the goodness-of-fit of the data to the two different
10 models defined by Eq. 4. and 4a.). For the data reported here the hypothesis that $C = 0$
11 cannot be rejected (P=0.30) and thus the assumption that labelled acetate addition doe not
12 stimulate the rate of methane production holds.

13 The contribution (%) that acetoclastic methanogenesis makes to total methane
14 production can be expressed in terms of the parameters of the fitted model as (B/A)*100%.
15 For the data reported here this gives a value of 66.3 (se 4.9) % (see Table 1). However, the
16 data also provide an estimate of the unlabelled acetate pool present in the microcosms. In
17 equations 1 and 2, the substrate concentration S is added labelled acetate and B is the
18 production rate of labelled methane when the acetate pool is, for all practical purposes,
19 comprised of labelled acetate only (i.e. the amount of labelled acetate is very large compared
20 to the indigenous unlabelled acetate pool). When the substrate pool comprises half labelled
21 and half unlabelled acetate, the production rate of labelled methane will be ½B. A property
22 of Michaelis-Menten kinetics is that the production rate is half its maximum when the
23 substrate concentration S equals K. It follows then that the estimate of K we obtain (Table 1)

1 is equivalent to the concentration of labelled acetate when it comprises half the total acetate
2 pool. On this basis it is clear that *K* provides a value for the amount of indigenous unlabelled
3 acetate present. For our data *K* (the in situ concentration of unlabelled acetate) was
4 estimated to be 62.3 (se12.8) µM which falls well within the concentration range typically
5 measured in aquatic ecosystems e.g. Lake Mendota (3.5 µM, Winfrey and Zeikus 1979),
6 Lake Vechten (5-6.7 µM, de Graaf *et al.* 1996), Lake Kinneret (25-55 µM, Nusslein *et al.*
7 2001; 20-100 µM, Duddleston *et al.* 2002). From this finding we can conclude that the
8 indigenous acetate pool was significantly increased (ca. 8 fold) in those microcosms which
9 had received most labelled acetate. It follows from this conclusion and the observation that
10 total methane production rates were the same irrespective of the amount of acetate added that
11 acetate was not rate limiting with respect to acetoclastic methanogenesis. This lack of
12 stimulation of methane production by acetate addition is indicative that acetate conversion to
13 CH_4 was occurring at, or near, the maximal rate (Winfrey and Zeikus 1979) in the sediments
14 studied here. These results present two alternative interpretations of the nature of acetoclastic
15 methanogenesis in the Rydal Water sediment. One interpretation is that, given the lack of
16 stimulation of methane production, the *Ks* (half saturation coefficient) values for the
17 acetoclastic methanogens present are lower (less than 62.3 µM) than the range reported for
18 cultured or enriched acetoclastic methanogens. *Ks* values determined for acetoclastic
19 methanogens enriched from sediment cores sampled from Lake Baldegger (Switzerland)
20 ranged from 1.3 to 6.75 mM depending on temperature (Lokshina *et al.* 2001). Those
21 enriched from an up-flow anaerobic sludge blanket (USAB) reactor exhibited *Ks* values from
22 6.4 to 8.8 mM (Lokshina *et al.* 2001) and *Ks* values for *Methanothrix* (*Methanosaeta)*-
23 dominated sludge and a pure culture of *Methanosarcina barkeri* had values of 0.69 mM and
24 5.57 mM respectively (Fukuzaki *et al.* 1990). The low apparent *Ks* value found for the Rydal

1 Water sediment may therefore reflect adaptation to low acetate environments. An alternative
2 interpretation of the data is that although the indigenous acetoclastic methanogens may have
3 *Ks* values comparable to that of cultured organisms they simply do not have the level of
4 enzyme expression when growing under limited acetate availability to respond to artificial
5 increases in the acetate pool over the short time frame of our experiments. For example, it has
6 been previously demonstrated (Dunfield and Conrad, 2000) in the cultured Type II
7 methanotroph *Methylocystis* strain LR1 that conditions of growth can alter apparent half-
8 saturation constants. Cells grown under starvation conditions had lower *Ks* values than those
9 growing in high methane environments. With this phenomenon in mind it can be speculated
10 that in other environments where the indigenous population are exposed to frequent and large
11 fluctuations in acetate pool size labelled methane production may rise instantaneously and
12 proportionately with the addition of acetate with no concomitant decrease in unlabelled
13 methane production. In these environments the Michaelis-Menten model would not apply and
14 separate measurement of the unlabelled acetate pool would be necessary to determine the
15 contribution acetoclastic methanogens to methane production.
16 The contribution of CO_2 reduction to methane production was independently
17 calculated from the ratio of $^{13}CH_4$ to $CH_{4\ total}$ and $^{13}CO_2$ to $CO_{2\ total}$ in microcosms amended
18 with $H^{13}CO_3^-$. As stated above this is possible because of the rapid equilibration of labelled
19 bicarbonate (in a well mixed slurry) which means that the measured ratio of labelled and
20 unlabelled CO_2 in the head space is equivalent to the ratio of heavy and light carbon in all
21 components of the inorganic carbon pool. This reasoning has been employed in numerous
22 studies (e.g. Conrad *et al.* 1989, Nüsslein *et al.* 2001), however in this study because a range
23 of different bicarbonate concentrations was used it was possible to determine the relationship
24 between the fraction of the bicarbonate that was labelled and the fraction of labelled methane

1 produced (Fig. 2). From this linear relationship the maximum contribution of CO_2 reduction
2 to total methane production (calculated from the slope and its standard error) was 28.9 (se
3 1.9) %. The intercept of the slope which is an estimate of the fraction of methane that is
4 labelled when no labelled substrate was added to the microcosm was 1.2 % (se 0.1). This
5 proportion agrees closely with repeated measurements of labelled and unlabelled methane
6 produced in un-amended sediment microcosms which, in turn, are in broad agreement with
7 the approximate and variable 1.1% natural abundance of ^{13}C-carbon in biologically derived
8 carbon (Yeh and Wang 2001).
9 The combined contributions of acetoclastic methanogenesis and CO_2 reduction to
10 total methane production derived independently using this novel approach (95.2% (se 6.8) are
11 consistent with these two pathways accounting for the majority of methane produced in these
12 anaerobic sediments. In addition, the relative ratios obtained ($CH_{4\ acetate}$: $CH_{4\ bicarbonate}$, 2.3 : 1)
13 are consistent with the theoretical contribution of these pathways ($CH_{4\ aceate}$: $CH_{4\ bicarbonate}$, 2 :
14 1) based on the production of H_2 and acetate from the anaerobic degradation of organic
15 matter (Conrad 1999).
16 The stable isotope titration method presented here represents a safe, simple and
17 relatively inexpensive way of estimating the contribution of different pathways of
18 methanogenesis to total methane production. However, we suggest that this titration approach
19 can also be used to investigate the degradation pathways and fate of other organic
20 compounds. Future work will develop titration methods to investigate the complex trophic
21 interactions involved in the anaerobic degradation of complex organic carbon in sedimentary
22 environments. We envisage that this approach when combined with Stable isotope analysis
23 (Radajewski *et al.* 2000), of RNA and DNA molecules which have become isotopically
24 labelled during microcosm incubations will provide a very powerful tool to understanding the

1 activities and interactions of individual members of the microbial consortia which mediate

2 carbon degradation.

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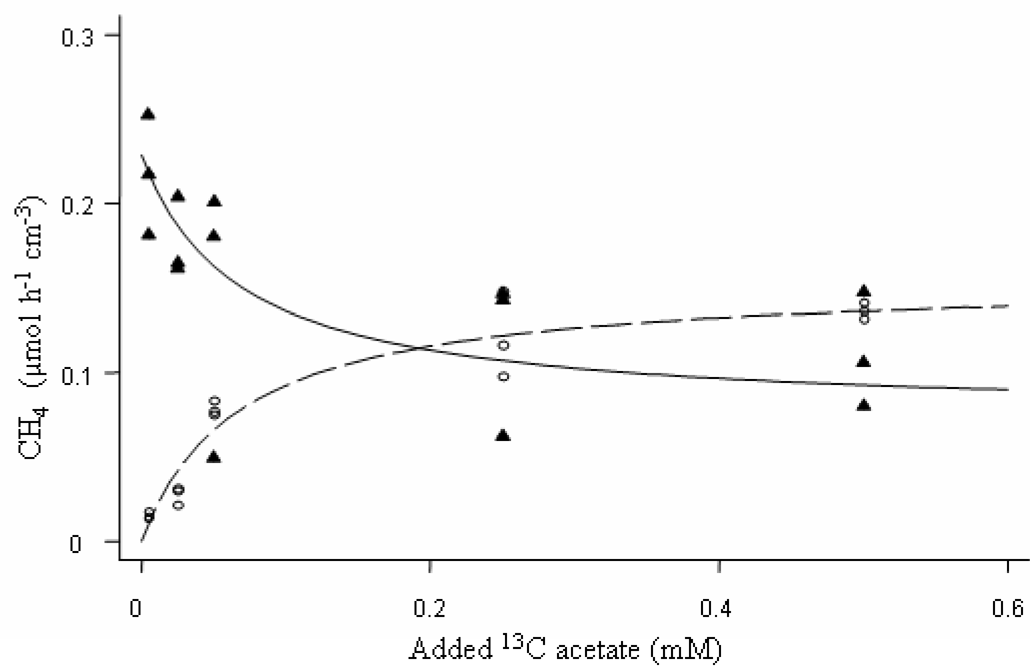
1 Figure legend:

2 Figure 1. Plot of unlabelled (▲) and labelled (○) methane production rates against added ^{13}C-
3 labelled acetate. The curves were derived by fitting (maximum likelihood) the raw data to the
4 model represented by equations 3 and 4.
5
6 Figure 2. Plot of the ratio of ^{13}C-labelled and total methane production against the ratio of
7 ^{13}C-labelled and total CO_2 in microcosms amended with $H^{13}CO_3$.

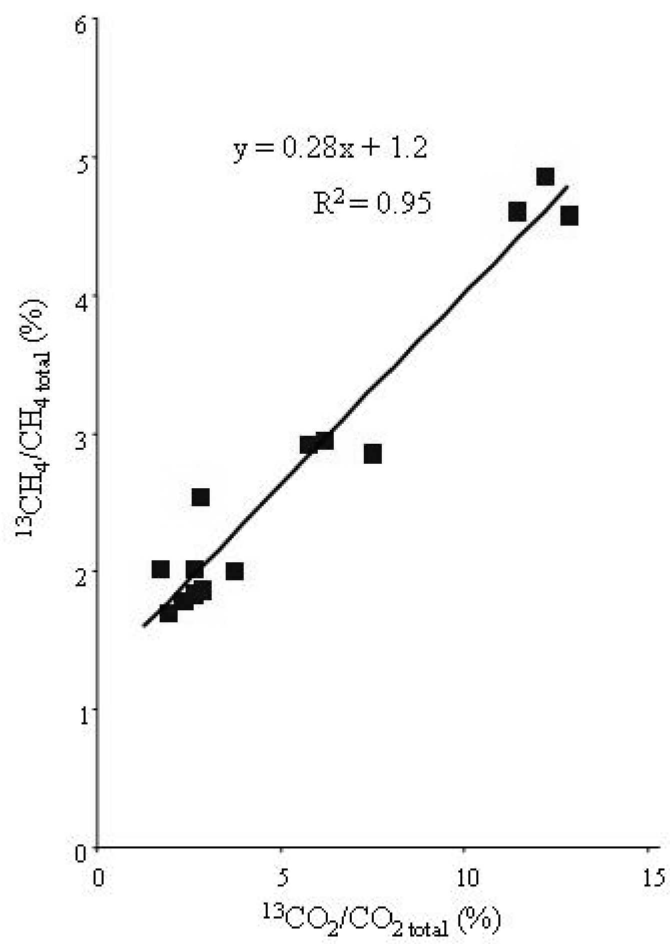
1 Figure 1.



2

1 Figure 2.

2



3

1 Table 1. Parameters derived from fitted (maximum likelihood) model

Parameter	
A	0.229 (0.0113)[a,b]
B	0.152 (0.0089)[a,b]
K	62.3 (12.8)[a,c]
σ_{12}	0.0418[d]
σ_{13}	0.0131[e]
Log-likelihood	71.98

2 [a] figures in brackets are standard errors.
3 [b] total CH_4 production rate (µmol h^{-1} cm^{-3})
4 [c] endogenous acetate concentration (µM)
5 [d] the residual standard deviation of $^{12}CH_4$ production rate (µmol h^{-1} cm^{-3})
6 [e] the residual standard deviation of $^{13}CH_4$ production rate (µmol h^{-1} cm^{-3})
7