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Deuterium as a quantitative tracer of enhanced microbial methane production

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ABSTRACT

Microbial production of natural gas in subsurface organic-rich reservoirs (*e.g.*, coal, shale, oil) can be enhanced by the introduction of amendments (*e.g.*, algal extracts from biofuel production) to stimulate microbial communities to generate "new" methane resources on human timescales, potentially providing a lower carbon energy source. This study tests deuterated water as a tracer to quantify the amount of "new" methane generated and the effectiveness of Microbial Enhancement of Coalbed Methane (MECoM) approaches, as methanogens incorporate hydrogen from formation waters into methane during methanogenesis. Microorganisms (including methanogens), formation water, and coal obtained from the Powder River Basin were used to establish batch reactor stimulation experiments, using algal extracts, in which incremental amounts of deuterated water were added. The greatest amount of methane was produced in the amended coal-associated experiments and there was a consistent uptake of D into microbial methane. The shorter duration (36 days) coal amended experiment had a lower slope (m = 0.31) of $\delta D\text{-}CH_4$ vs. $\delta D\text{-}H_2O$ and a similar offset between $\delta D\text{-}H_2O$ and $\delta D\text{-}CH_4$ (371.2‰) compared to the longer duration (m = 0.44; 114 days; 358.8‰ offset) experiment, both consistent with the stimulation of primarily acetoclastic methanogenesis. The success of our proof-of-concept laboratory experiments confirms that deuterated water can be used as a quantitative tracer of stimulated coal-associated methanogenic activity. We also provide an example of how it can be applied in field-scale MECoM projects. In addition, deuterated water may serve as a useful tracer for other natural or enhanced subsurface microbial activities, such as microbial enhanced oil recovery or bioremediation of organic contaminants.

1. Introduction and background

Natural gas currently accounts for approximately 30% of the United States' current energy production, and as global demand for energy has increased, natural gas production has been projected to continue to increase or plateau to meet a projected increase in demand [1]. One proposed method for boosting current natural gas production in shallow coalbeds, particularly in depleted gas reservoirs [2] is Microbial Enhancement of Coalbed Methane (MECoM; [3]). This method can provide a lower carbon emitting energy source, when MECoM is coupled to algae growth for biofuels in coalbed methane produced water storage ponds, which consumes atmospheric CO_2, and algal extracts are used as the stimulant for MECoM [4].

Methane derived from anaerobic microbial degradation of long-chain hydrocarbons has long been recognized as a significant fraction of the natural gas produced in thermally immature coalbeds [5]. As these microbial communities are actively producing methane in low rank coals (*e.g.*, Powder River Basin (PRB); [6]), the aim of MECoM is to stimulate *in situ* microbial communities to accelerate the natural conversion of coal to methane. Stimulation of these microbial communities is important to consider as the natural accumulation of commercially viable volumes of methane *in situ* may take millennia [7,8]. Non-

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stimulated laboratory rates of microbial methanogenesis may be much faster, compared to natural, non-stimulated field rates, although they still occur on month to century time scales [9,10]. Recent studies investigating strategies for stimulating subsurface methanogenic communities have shown that coal to methane conversion can be enhanced by the pre-oxidation of coal [11,12] and addition of amendments (e.g., algal and yeast extracts) in laboratory experiments (e.g., [4,13–15]). However, there is no broadly accepted method for quantifying the outcome of field-scale stimulations to compare stimulants or to test the success of MECoM regardless of the stimulant used.

Most previous field-scale MECoM projects used established coal seam wells, injected water mixed with a stimulant into a coal bed and sealed the well for the test duration (Fig. 1A). After a set incubation time, the wells were pumped to recover the methane presumably generated in part by the stimulation. These stimulations relied on gas production curves generated from nearby wells to estimate the amount of methane produced as a result of stimulation [3,16]. This method attributes all methane produced after stimulation in excess of that predicted by the production curve to the stimulation (Fig. 1B) and does not account for external factors that may lead to greater methane production. Such factors may include: methane released by shutting in the well, spatial variation in nearby wells, or degassing of methane sorbed to the coal as a result of stimulant injection.

More recent field-scale MECoM projects have installed new wells equipped with *in situ* methane sensors to monitor changes in methane concentrations during stimulation experiments [17]. If there is a large background concentration of methane in the coal beds, however, it may be difficult to detect a proportionally small increase in methane concentrations as a result of MECoM efforts. This study evaluates the feasibility of using deuterium-enriched water as a more sensitive and quantitative tracer of "new" methane generation during MECoM through proof-of-concept laboratory stimulation experiments.

Valentine (2015) [18] patented the general idea of using deuterium as a tracer of "new" methane generated as a result of manipulation of sub-surface environments. However, the concept was only tested using a co-culture of *Syntrophothermus lipocalidus* and *Methanothermobacter thermautotrophicus* to degrade butyrate. Valentine (2015) [18] did not use coal as a substrate, *in situ* coal-associated methanogenic cultures, or common MECoM amendments, such as algal or yeast extracts [13], in their proof-of-concept experiment. To our knowledge, there are no laboratory experiments or field-scale tests verifying deuterated water as a tracer of "new" methane for MECoM applications. Successful results of our initial laboratory experiments demonstrate that deuterium-enriched water can be used to quantify the efficacy of MECoM efforts and can be easily scaled for application in the field.

1.1. Coal biodegradation and microbial methane generation

Methane produced through the syntrophic degradation of coal is characterized by the enzymatic breakdown of coal into soluble organic intermediates (small polyaromatic hydrocarbons, long chain fatty acids, ketones, and a variety of others). These intermediate molecules are then converted into the simple organic compounds (e.g., acetate, formate, methanol) and inorganic molecules (CO_2) used for methanogenesis [5,19–23]. Stable isotopes can be useful indicators of the specific pathway used for microbial methanogenesis [23,24]. In anaerobic environments generally, three pathways (hydrogenotrophic, acetoclastic, and methylotrophic) have been observed to dominate methanogenic growth [25].

$$\text{Hydrogenotrophic}: 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

$$\text{Methylotrophic}: 4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (2)$$

$$\text{Acetoclastic}: CH_3COOH \rightarrow CH_4 + CO_2 \quad (3)$$

The hydrogenotrophic pathway utilizes carbon dioxide and hydrogen gas to produce methane. Because the dissolved hydrogen gas (H_2) used in hydrogenotrophy has been interpreted to equilibrate rapidly and fully with intracellular water [26,27], the deuterium content of the methane produced from this pathway should reflect that of the water present *in situ* at a 1:1 uptake ratio [28]. In contrast to this, the two other methanogenic pathways, methylotrophic and acetoclastic, can both produce methane using an exogenous methyl group derived from organic matter. Consequently, the deuterium content of the methane resulting from these pathways exhibits at least a 1:4 mixing ratio with the *in situ* water [29].

These differences in the mixing ratios among methanogenic pathways have been used to identify the predominant methanogenic pathway in field-based studies [23], such as the Powder River Basin [30]. Hydrogen isotope measurements of CH_4 and H_2O sampled from the Powder River Basin could suggest that a majority of coalbed methane in most of the basin is produced through hydrogenotrophic methanogenesis [23,30–32]. However, microbial community analyses have suggested active hydrogenotrophic, methylotrophic, and acetoclastic methanogens in this and other coal basins [22,33–35]. One possible explanation for this apparent contradiction may be the

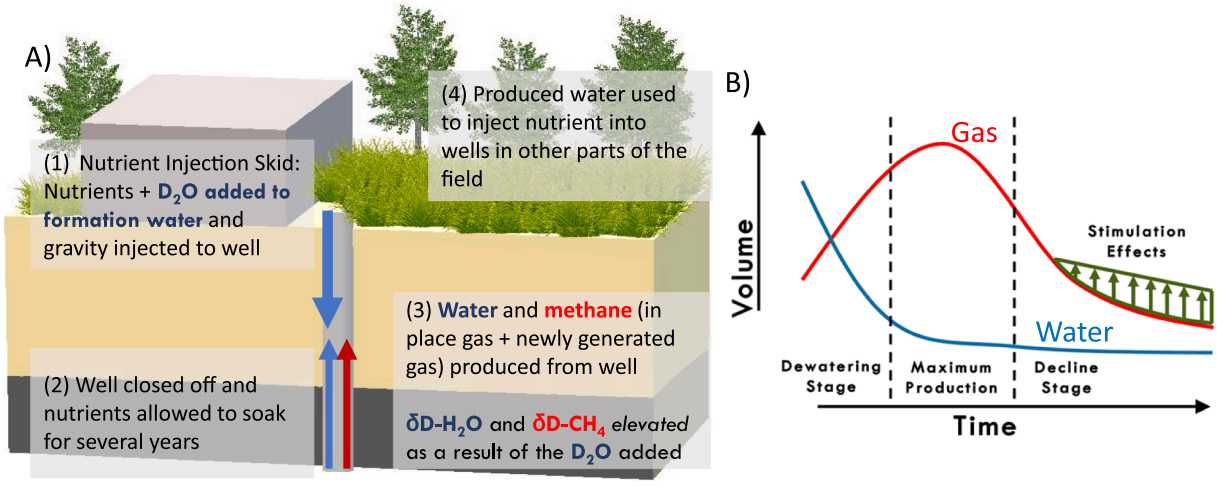

Fig. 1. A) Infographic representation of the idealized steps for field-scale enhanced microbial coalbed methane production (MECoM). Addition of D_2O to the stimulant injection allows for quantification of 'new' methane generated based on the change in the $\delta D\text{-}CH_4$ values of the *in situ* methane before and after the stimulation. B) Idealized gas production curve, which gives a qualitative measure of the increase in gas production after a stimulation. Modified from Ritter et al. (2015a) [3] and Nuccio (2000) [16].

equilibration of hydrogen between the *in situ* water and the precursor molecules of acetoclastic and methylotrophic methanogenesis, which can occur as a result of relatively slow rates of methanogenesis in organic-rich reservoirs [23,36]. Conversely, this phenomenon could also be explained by substrate constraints that limit the amount of methane produced by acetoclasts and methylotrophs as compared to hydrogenotrophic production. While this remains an important issue when translating experimental findings to field-scale understanding, hydrogen isotope equilibration with water is not expected to pose a significant problem to the described batch study in which substrates are abundant, the reaction rates are relatively fast, and incubation periods are relatively short compared to the natural environment.

Acetate concentrations have been observed to build up during the early stages of laboratory MECoM stimulations prior to rapid depletion concurrent to a rapid increase in the methane concentration, suggesting that acetoclastic methanogens may be the dominant producers of methane during such stimulations (e.g., [11,37]). Indeed, quantitative PCR (qPCR) using 16S rRNA selective primers performed during these stimulations showed that the preponderance of 16S rRNA present during the peak of methane production corresponded to the genus *Methanosaeta*, an obligate acetoclast [11]. Similar MECoM experiments, using various amendments, including algal extracts, showed an increase in methane production and the predominance of *Methanosaeta* spp. regardless of the stimulant used [13]. Thus, we expected that acetoclastic methanogenesis would be the dominant methanogenic pathway in our laboratory incubation experiments of stimulated methanogenesis using algal amendments. It is intriguing that Davis et al. (2018) [13] found a relatively high abundance of the genus *Methanospirillum*, a known hydrogenotrophic methanogen, in unamended coal MeCoM experiments. Jones et al. (2010) [11] also found members of the order *Methanomicrobiales* (known non-acetoclastic methanogens) to be dominant early in their experiments before peak methane production. Thus, we hypothesized a shift in methanogenic community from acetoclastic to more hydrogenotrophic methanogens following depletion of amendments.

1.2. Deuterated water as a tracer of methanogenesis

Deuterated water has previously been used as a tool to estimate metabolism in pathogens [38], determine the origins of hydrogen in lipid biosynthesis [39], determine microbial activity patterns [40], quantify bioremediation [41], and has recently been used with varying degrees of success as a method to quantify non-stimulated methanogenesis [10,42,43]. It is important to note that all the methanogenic pathways either directly use water as a hydrogen source and/or incorporate organic hydrogen that has had the opportunity to equilibrate with water. Thus, we hypothesized that deuterium enrichment of the *in situ* water will result in predictable enrichment of the methane produced during the stimulation. Further, we expect to be able to translate this enrichment of methane to quantify field scale stimulation effectiveness through a mixing model (Equation (4)).

$$V_{new} = \frac{(R_{mix} - R_{old})}{R_{new} - R_{mix}} \times V_{old} \quad (4)$$

Where: V_{new} = the amount of "new" methane generated as a result of the stimulation

R_{old} = the isotopic ration of deuterium of the *in situ* methane prior to stimulation

R_{new} = the isotopic ratio of deuterium of the methane produced during the stimulation

R_{mix} = the isotopic ratio of deuterium in methane produced from the well after the stimulation; reflects the mixing of the two end members

V_{old} = the amount of methane present *in situ* prior to stimulation

As the amount of methane present *in situ* prior to the stimulation may not be known, a more general form of the mixing model can be given such that V_{new} is expressed as a multiple of V_{old} (Equation (5)).

$$rV_{new} = \frac{(R_{mix} - R_{old})}{R_{new} - R_{mix}} \quad (5)$$

where: rV_{new} = the amount of "new" methane generated as a result of the stimulation relative to the amount present *in situ* prior to stimulation.

2. Methods

To test the utility of deuterated water to quantify "new" methane generated during MECoM, subsurface conditions in the PRB were replicated in two benchtop stimulation experiments. These experiments were conducted using varying mixtures of 99.99% D_2O and *in situ* PRB water (Table 1) in addition to algal amendments (SLA-04) following the same protocols used by Davis et al. (2018) [13]. The first experiment (Exp1) was run to verify the uptake of deuterium in the CH_4 produced as a proof of concept. The second experiment (Exp2) served to greatly extend the range of $\delta D\text{-}CH_4$ values generated by Exp1, by mixing significantly more 99.99% D_2O with the PRB water.

2.1. Site description and sample collection

Coal cores were collected in July 2013 from the sampling site near Birney, Montana (USA), previously described by Barnhart et al. (2016) [6], during the drilling of two new wells in the Flowers-Goodale (FG) coalbed. The FG coalbed is Paleocene in age and of low rank (subbituminous) and low sulfur content [30]. At the Birney site, the FG coalbed is located between approximately 112 to 120 m depth. The 2-inch diameter, 12-inch length cores were stored in polyvinyl chloride (PVC) tubes and filled with formation water pumped from the FG-11 well. Water was collected in six-gallon plastic storage jugs from the FGM-13 well in April 2016 and stored at 4 °C upon return to the laboratory (Montana State University, Bozeman, MT) until experiment setup. Microbial cultures were collected in September 2015 from the FGP-13 (Exp 1) and FGM-13 (Exp2) wells, screened in the Flowers-Goodale coalbed and located approximately 25 m apart, using the Diffusive Microbial Sampler (DMS) previously described [33]. Five mL of slurry from the DMS were added to a previously prepared serum bottle with 5 g of FG coal, 45 mL anoxic FG formation water, and 5% CO_2/95% N_2 headspace before being incubated in the dark at room temperature (21 °C ± 1) until use as inoculum in the studies described here.

2.2. Amendment growth and processing

The algal amendment was grown and processed as previously described [13]. In short, the *Chlorella* sp., strain SLA-04 was cultivated in Bold's Basal Medium in tube photobioreactors. The algal biomass was concentrated by centrifugation and lyophilized. The dried biomass was ground with a mortar and pestle and mixed at 10x desired concentration (0.5 g/L final concentration) in degassed FG formation water.

2.3. Batch growth

The tube microcosms for both experiments were set up in 26-mL Balch tubes sealed with butyl rubber stoppers and aluminum crimp seals. The FG coal core was opened in an anaerobic glove bag where it was manually crushed, sieved for uniform size distribution (0.85–2 mm), washed with deionized water to remove grains finer than 0.85 mm, and dried before it was added to the Balch tubes. One-mm borosilicate glass beads (GB) were autoclaved to be used in lieu of coal for controls. Each Balch tube received 1 g of coal or GB. A large volume of FG formation water was sparged overnight with a 5% CO_2:95% N_2 gas mixture and reduced with sulfide (1 mM as $Na_2S\text{-}9H_2O$). The FG water was added to the Balch tubes using anoxic techniques in volumes such that the total liquid volume of each microcosm reached 10 mL (Table 1).

Table 1
Summary of deuterated water experiments using coal and glass beads (control), with and without algal extract amendments (SLA-04), and with and without native Powder River Basin coal-associated microbial communities. Each experiment was performed in triplicate.

Sample ID [n = 3]	Inoculum [mL]	Solid Media [1 g]	SLA-04 [mL]	CBM Water [mL]	99.9% D_2O Added [uL/L]	Initial δD-H_2O [‰] (VSMOW)	Final δD-CH_4 [‰] (VSMOW)	CH_4 Produced at End of Experiment [μg CH_4/g coal or glass beads]
Exp1 – 36 days								
D1	1	Coal	1	8	0.0	−133.8	−411.9 ± 3.1	372.2 ± 79.3
D2	1	Coal	1	8	23.4	16.2	−367.6 ± 5.0	291.5 ± 92.6
D3	1	Coal	1	8	31.2	66.2	−350.5 ± 3.1	230.3 ± 74.4
D4	1	Coal	1	8	39.0	116.2	−333.7 ± 4.2	289.1 ± 60.0
D5	1	Coal	1	8	46.8	166.2	−318.3 ± 2.0	348.5 ± 122.4
Exp2 – 114 days								
C1	0	Coal	0	10	0.0	−125.5	(ND*)	0
C2	1	Coal	0	9	0.0	−125.5	−398.3 ± 2.1	328.9 ± 36.4
C3	1	Glass Beads	0	9	0.0	−125.5	−394.0 ± 7.4	301.2 ± 40.1
C4	0	Coal	0	10	20.8	8.3	−384.7 ± 1.4*	7.6 ± 7.1*
C5	1	Coal	0	9	20.8	8.3	−344.2 ± 1.6	336.4 ± 59.7
C6	1	Glass Beads	0	9	20.8	8.3	−349.2 ± 7.1	307.2 ± 19.0
T1	1	Coal	1	8	0.0	−125.5	−418.3 ± 2.0	788.5 ± 74.0
T2	1	Glass Beads	1	8	0.0	−125.5	−437.2 ± 8.3	61.0 ± 3.8
T3	1	Coal	1	8	20.8	8.3	−361.4 ± 0.5	942.7 ± 84.3
T4	1	Glass Beads	1	8	20.8	8.3	−365.9 ± 6.3	73.7 ± 12.3
E1	1	Coal	1	8	98.8	508.3	−161.3 ± 1.4	859.0 ± 95.5
E2	1	Coal	1	8	176.8	1008.3	85.9 ± 6.3	892.9 ± 90.6
E3	1	Coal	1	8	254.8	1508.3	315.1 ± 17.8	883.6 ± 64.9

(ND*) – δD-CH_4 was not determined due to insufficient CH_4 present in the gas sample.
* no methane was detected by gas chromatography at Montana State University, however very small quantities of methane were detected at University of California at Davis during isotopic analysis and reported here.

To establish a consistent fractionation line, increasing volumes of 99.99% D_2O were added to the FG water (Table 1). When it was not possible to accurately pipette very small volumes of 99.99% D_2O, serial dilutions were used to achieve the desired concentration. The tubes were sealed with a 5% CO_2/95% N_2 headspace. The algal amendment and inoculum were prepared as described above. All amended treatments received 1 mL of this prepared amendment concentrate and all inoculated microcosms received 1 mL of the prepared inoculum slurry (Table 1). All treatments were incubated in the dark at room temperature (21 ± 1 °C) for the duration of the experiment. Exp1 was only run until head space methane was detected in high enough concentrations to show methanogenic growth (36 days), while Exp2 was run until the head space methane concentration no longer showed significant increases (114 days) (Fig. 2).

2.4. Gas and water analyses

To verify methanogenic growth, 1-mL gas samples were drawn monthly from the Balch tube head space and analyzed as previously described [13]. To replace the sampled gas volume, the volume of head

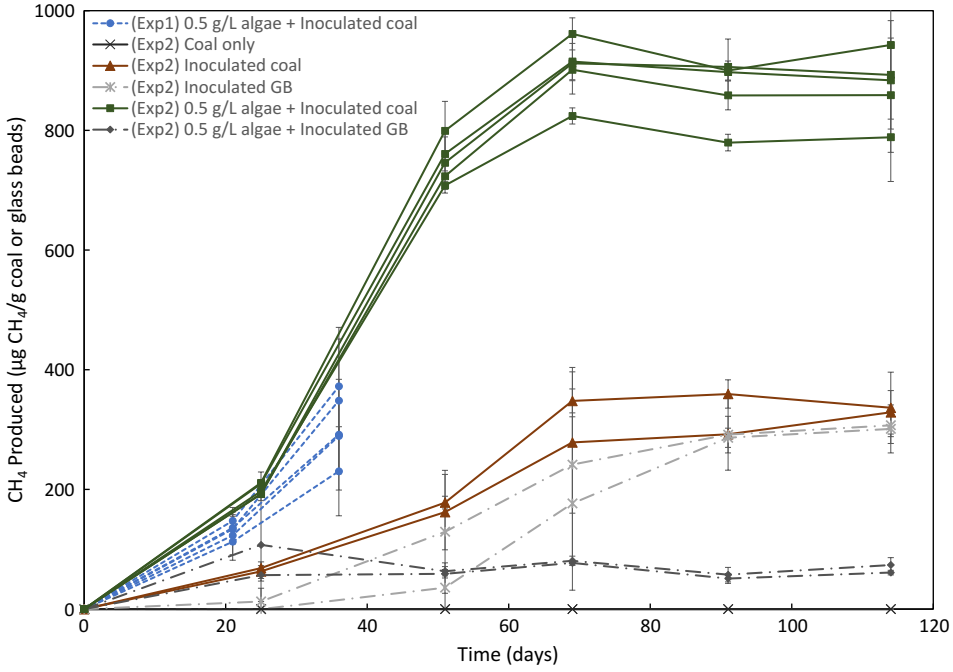

Fig. 2. Plot of methane production over time in amended and unamended experiments with coal or glass beads for Exp 1 (36 days) and Exp 2 (114 days). Cumulative average methane concentrations are shown with error bars for one standard deviation for triplicates of each treatment.

space drawn from the samples was replaced by sterile 1 mL of a 5% CO_2/95% N_2 gas mixture. The gas composition of each sample was determined via gas chromatography at Montana State University following the protocols previously detailed [13]. At the end of each experimental period, 1 mL headspace gas was collected via a gas tight syringe, injected into 12-mL Exetainer® vials (Labco, Lampeter, UK) filled with N_2 at 1 atm to slightly overpressure the vials, and sent with 12 mL of initial formation water samples to the University of California at Davis Stable Isotope Facility for H isotope analysis on a continuous flow Isotope Ratio Mass Spectrometer [44]. H isotope measurements were within 1% of calibration standards.

3. Results and discussion

Time series analysis of the head space gas present in each microcosm confirmed elevated methane production for all amended samples with coal as a substrate, as compared to unamended samples and amended samples without coal, as previously demonstrated by our group [13] (Fig. 2). Very little to no methane was detected in the microcosms that only contained coal or glass beads that did not have inoculum present (Table 1). Microcosms containing inoculated coal or glass beads without any amendments produced an average of 318.8 g CH_4/g coal or glass beads. The highest methane production was observed in inoculated microcosms containing algae amendment and coal (average 865.6 g CH_4/g coal), approximately 2.7 times more than the unamended inoculated coal or glass bead treatments.

The first experiment (Exp1), run for 36 days with coal, with inoculum and amendments, demonstrated a predictable incorporation of deuterium from the labeled water into the methane as a function of the deuterium concentration in the water (Fig. 3). The headspace methane collected at the end of Exp1 had δD-CH_4 values that ranged from −411.9 ± 3.1‰ to –318.3 ± 2.0‰ (VSMOW, Vienna Standard Mean Ocean Water), corresponding to initial δD-H_2O values that ranged from −133.8 to 166.2‰ (VSMOW), respectively (Table 1). We observed a consistent offset between δD-H_2O and δD-CH_4 ($R^2 = 0.9872$), with a slope of 0.31, as described by the following equation:

$$\delta^2 H_{CH4} \pm 2.8 = 0.31\left(\delta^2 H_{H2O} \pm 0.6\right) - 371.2 \quad (6)$$

There were no Rayleigh-like fractionation effects observed during the experiments because the water was greatly in excess compared to the CH_4.

The second experiment (Exp2) was designed to more accurately simulate the D_2O concentrations and timeframe of a field-scale stimulation. Thus, the microorganisms were allowed to incubate until methane production was observed to reach stasis (114 days). The headspace methane from the amended coal treatments with inoculum in Exp2 showed δD-CH_4 values that ranged from −437.2 ± 8.3‰ to 315.1 ± 17.8‰ (VSMOW), corresponding to initial δD-H_2O values that ranged from −125.5‰ to 1508.3‰ (VSMOW), respectively (Table 1 and Fig. 3). We observed a consistent linear offset between δD-H_2O and δD-CH_4 ($R^2 = 0.997$), with a slope of 0.44, indicative of a consistent mode of deuterium incorporation described by:

$$\delta^2 H_{CH4} \pm 1.7 = 0.44\left(\delta^2 H_{H2O} \pm 0.8\right) - 358.8 \quad (7)$$

The data from amended coal treatments in Exp1 and Exp2 were then plotted in conjunction with each other, the expected hydrogen isotope fractionation lines of both hydrogenotrophic and acetoclastic/methylotrophic methanogenesis [28,45], and co-produced formation water and gas data from PRB coalbed methane (CBM) wells [3,6] (Fig. 3).

In addition to the amended coal treatments in Exp2, a series of controls were run in parallel to determine the origin of the hydrogen molecules incorporated into the methane produced during Exp2 (Table 1). The δD-CH_4 values measured at the end of the experiments could show hydrogen molecules originating from three possible sources: 1) methane that was initially sorbed to the inoculum or coal at the beginning of the experiments, 2) covalently bound hydrogen in the organic material present in each microcosm, or 3) the water present in each microcosm. If a significant amount of the final methane volume in our experiments originated as naturally-formed microbial gas that desorbed during the experiments, we would expect the δD-CH_4 values would resemble those measured at the field site. However, microcosms that only contained coal showed: A) very little or undetectable methane at the end of the experiment, and B) the small amounts of measurable methane were not isotopically similar to methane present in the field. The general lack of methane desorbing during the experiments and its

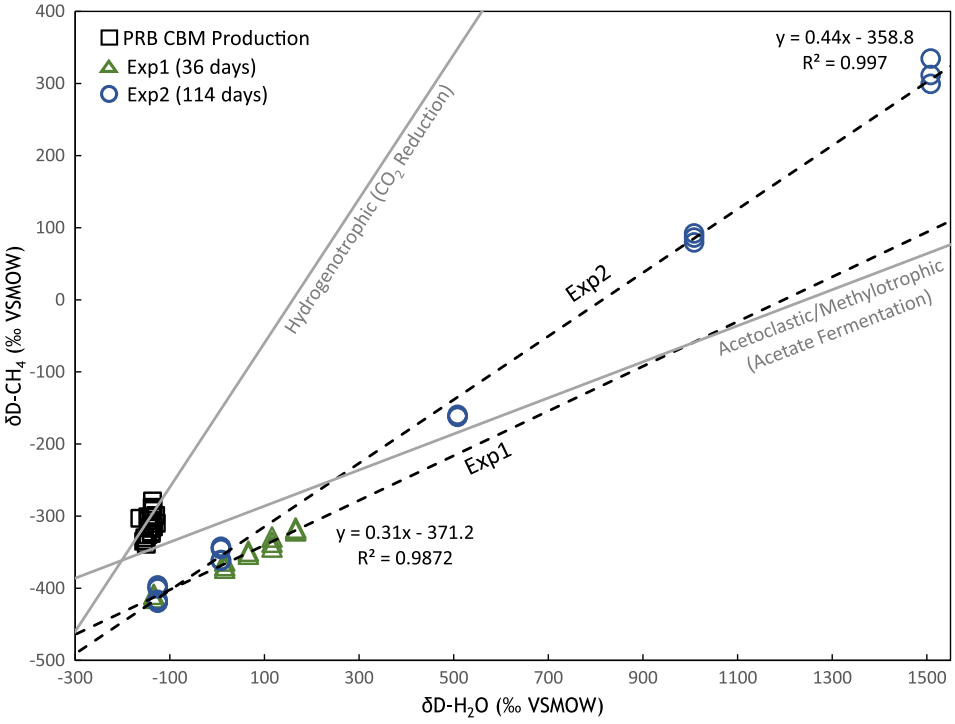

Fig. 3. Plot of the δD-CH_4 vs δD-H_2O values for the second experiment (Exp2), relative to the first experiment (Exp1), and Powder River Basin (PRB) coalbed methane (CBM) production well data [3,6]. Note the tight grouping of each replicate and the similar δD-CH_4 values of Exp1 and Exp2 as compared to PRB CBM production well data at the same δD-H_2O value. It is also important to note the higher slope in Exp2 (0.44) versus Exp1 (0.31), which suggests mixing between acetoclastic/methylotrophic and hydrogenotrophic methanogenesis. Although the δD values in Exp2 exceed the range of normal environmental conditions, the effect on mixing using delta notation is small (error within symbol size).

isotopic dissimilarity to methane present at our field site strongly suggest that there was very little or no hydrogen originating from sorbed methane in our amended coal treatments. Furthermore, there was a strong dependence of the δD-CH_4 values measured at the end of Exp2 on the δD-H_2O value in each treatment, indicating that the primary control of the δD-CH_4 value for the experimental treatments was the δD-H_2O values of the water.

Two notable trends were observed from Fig. 3: 1) the methane produced using the *in situ* PRB water was significantly depleted in deuterium, even when D_2O was not added to the formation water (as shown by the large decrease in the δD-CH_4 value as compared to the PRB data); and 2) the relatively low slope of the linear regressions (m = 0.31 and 0.44 for Exp1 and 2, respectively) is similar to the expected values for pure culture acetoclastic/methylotrophic methane production (m = 0.212–0.269) mixed with a smaller amount of hydrogenotrophic methane (m = 0.571) [46].

The more negative δD-CH_4 values of experimental samples compared to field data can be explained as the result of kinetic fractionation during microbial methanogenesis. This kinetic fractionation has been regularly observed by others [16,27,28] and has been explained by a low degree of reversibility during the enzymatic production of methane [46,47]. Furthermore, there is evidence of a preference for the acetoclastic/methylotrophic pathway, supported by the slopes of the linear regressions and offset between δD-H_2O and δD-CH_4 (371.2‰ for Exp1; 358.8‰ for Exp2), which are similar to the mixing ratio expected from acetoclastic/methylotrophic methanogenesis (>300‰ offset; [28]), as well as experimentally determined values [27,29,46].

The offset between δD-H_2O and δD-CH_4 indicative of acetoclastic methanogenesis is consistent with microbial analysis of previous MECoM experiments conducted by our group [13], using the same experimental setup (e.g., type and concentration of amendments, coal cores, formation waters, and microbial cultures) without the addition of deuterated water. The previous study showed the archaeal community in the amended coal experiments was dominated by *Methanosaeta* (obligate acetoclasts). These results are also consistent with other previous studies showing acetoclastic and/or methylotrophic methanogenesis dominating stimulated methane production in the laboratory [11] and pilot MECoM field studies [48].

When comparing the two experiments, the two emerging trends that were present in Exp1 were also present in Exp2. The most striking difference between the two experiments is the difference in the slope of the δD-CH_4 versus δD-H_2O values. With a lower slope for Exp 1 (m = 0.31), the uptake of deuterium revealed in the headspace methane from Exp2 (m = 0.44) still resembles acetate fermentation and/or methylotrophic methanogenesis (Fig. 3), but may be indicative of a greater degree of mixing between methane derived from acetoclastic and/or methylotrophic (earlier), and hydrogenotrophic (later) pathways. We hypothesize that this pathway-mixing occurs during the longer time period compared to Exp1 due to substrate limitations (i.e., depletion of amendments) and H_2 concentrations reaching threshold values for hydrogenotrophic methanogenesis over the longer experimental time [24]. This is consistent with the higher relative abundance of hydrogenotrophic methanogens, in addition to the predominance of acetoclastic methanogens, observed in our group's previous experiments of unamended coals [13]. In addition, this is consistent with the shift observed by Jones et al. (2010) [11] from more hydrogenotrophic methanogenesis early in MECoM experiments, prior to peak acetate depletion and methane production when acetoclastic methanogenesis was dominant.

It is unlikely the difference in slope between Exp 1 and Exp 2 (Fig. 3) can be explained by differences in microbial culture sample sources. The microbial culture for Exp 1 was from a diffusive microbial sampler installed in a different well (FGP-13) than Exp 2 (FGM-13). However, both wells were screened in the Flowers-Goodale coal and are in close proximity. Previous characterization of the microbial communities between the two microbial culture samples from the different wells showed no apparent difference in amended coal experiments [13].

The higher slope for Exp 2 may reveal some degree of H isotope exchange between the CH_4 and water over the longer duration experiment (114 days); however, timescales of both experiments were still relatively short compared to residence times of typical groundwaters in CBM systems (tens of years to hundreds of thousands of years) where H isotope exchange has been observed [31,49]. Alternatively, the slope difference between the two experiments could be an artifact of extrapolating the fractionation line for the first experiment to much higher δD values than was actually measured.

The information gathered from these two experiments may be used as a useful tool in field scale MECoM to quantify the methane generated as a direct result of the stimulation. For example, the clustering observed in the δD-CH_4 values of each replicate implies reproducibility of the results and builds the case for the predictability needed to validate that the deuterium content of the methane produced as the result of a MECoM stimulation reflects the deuterium content of the *in situ* water. As noted above, the current stimulation method involves the injection of a water-mixed stimulant (or amendment). After determining the baseline δD-H_2O values of both the injection and formation water, and baseline methane flux from the well, D_2O can be added to the injection water to change the δD-H_2O value to a target value [18]. This target value would be determined by the measured δD-CH_4 value of the "old" methane prior to MECoM, and a calculation of the desired δD-CH_4 value of the "new" methane (Eqn (5)). Upon injection of the water-mixed stimulant, the well may be closed for the duration of the stimulation. After MECoM, the δD-CH_4 value of the produced methane should reflect the mixing of methane produced during the stimulation and the baseline methane present *in situ* prior to stimulation. The production of "new" methane during the stimulation can then be determined according to Eqn (5). For example, if 250 L of water is added with amendments to a well for MECoM, 100 mL of deuterated water added would be enough to increase the δD-H_2O value of the injected water (>2400‰) and the formation water around the well (mixture of injected water and ambient formation water) to >700‰. Using the linear regression line for Exp2 (Equation 7), assuming stimulation of a similar mix of methanogenic pathways in the field as in our laboratory experiments, an initial δD-H_2O value of 700‰, and an initial δD-CH_4 value similar to that found in Powder River Basin coal beds (-300‰), we would predict a resultant δD-CH_4 value of −54‰ of the "new" methane, and a δD-CH_4 value of −152‰ for the total ("new" and "old") methane produced at the end of the experiment if 1.5 times more "new" methane was generated compared to the "old" methane in place.

When considering that roughly half of all organic carbon degraded by anaerobic microbes is eventually converted to methane [50], with the actual fraction of organic matter converted to methane dependent on the carbon source [23], the importance of quantifying methanogenesis becomes apparent. Further, the use of deuterated water as a quantitative tracer is applicable beyond MECoM, as methanogenesis is only one of the possible pathways in which subsurface microorganisms integrate water-based hydrogen into molecules of interest. Current understanding of subsurface carbon cycling is limited, as it involves the syntrophic pairing of many microbial processes [23,34]. Many of these microbial processes compete for limited nutrients, and the insight gained by using deuterated water to quantify stimulated methanogenesis may be useful for quantifying the stimulation of other subsurface carbon cycling pathways.

Adding deuterated water, or other isotopic tracers like tritium or labeled carbon substrates, to stimulated carbon cycling processes provides the capability of quantifying these processes, even when large background concentrations of the products exist. This can be done economically, as the relatively low deuterium content of most natural waters is easily changed by small additions of D_2O. One such example may lie in bioremediation, as the addition of D_2O may be useful in quantifying the results of bioremediation efforts in large spills and in areas where products of biotransformation are already present in large

concentrations.

4. Conclusions

Consistent uptake of deuterium was observed during the laboratory stimulation of coal-dependent methanogenesis with algal extracts, which was highly correlated with the initial deuterium content of the water. Success of the proof of concept MECoM experiments shows promise for the quantification of stimulated coalbed methanogenesis in the field using deuterated water as a tracer and may also aid in differentiating between different methanogenic pathways. The distinct hydrogen isotope fractionation trend seen in the laboratory stimulation experiments coincides with a previous experiment using the same coal substrates, formation waters, microbial cultures and amendment, demonstrating the predominance of sequences indicative of acetoclastic methanogenesis, as seen in other laboratory and field MECoM stimulation experiments. This is in contrast to natural CBM field conditions where hydrogenotrophic, methylotrophic and acetoclastic methanogenesis have been observed. Addition of deuterated water has applicability as a tracer beyond quantifying MECoM and may be a useful tool in tracing the stimulation of other subsurface carbon cycling pathways.

CRediT authorship contribution statement

Kilian Ashley: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. **Katherine J. Davis:** Methodology, Investigation, Writing - review & editing. **Anna Martini:** Methodology, Formal analysis, Writing - review & editing. **David S. Vinson:** Formal analysis, Writing - review & editing. **Robin Gerlach:** Supervision, Methodology, Writing - review & editing. **Matthew W. Fields:** Supervision, Methodology, Writing - review & editing. **Jennifer McIntosh:** Supervision, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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