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Investigations of Aerobic Methane Oxidation in Two Marine Seep Environments Part 2: Isotopic Kinetics

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Key Points:

- Systematic seawater incubations were conducted to determine stable isotopic changes associated with aerobic methane oxidation.
- Isotopic fractionation caused by aerobic methane oxidation was shown to follow first-order isotopic kinetics.
- Despite displaying a microbial bloom associated with rapid oxidation events, the isotopic fractionation factor remained constant.

Abstract

During aerobic oxidation of methane (CH_4) in seawater, a process which mitigates atmospheric emissions, the ^{12}C-isotopologue reacts with a slightly greater rate constant than the ^{13}C-isotopologue, leaving the residual CH_4 isotopically fractionated. Prior studies have attempted to exploit this systematic isotopic fractionation from methane oxidation to quantify the extent that a CH_4 pool has been oxidized in seawater. However, cultivation-based studies have suggested that isotopic fractionation fundamentally changes as a microbial population blooms in response to an influx of reactive substrates. Using a systematic mesocosm incubation study with recently collected seawater, here we investigate the fundamental isotopic kinetics of aerobic CH_4 oxidation during a microbial bloom. As detailed in a companion paper, seawater samples were collected from seep fields in Hudson Canyon, US Atlantic Margin and atop Woolsey Mound (a.k.a. Sleeping Dragon) which is part of lease block MC118 in the northern Gulf of Mexico, and used in these investigations. The results from both Hudson Canyon and MC118 show that in these natural environments isotopic fraction for CH_4 oxidation follows a first-order kinetic process. The results also show that the isotopic fractionation factor remains constant during this methanotrophic bloom once rapid CH_4 oxidation begins and that the magnitude of the fractionation factor appears correlated with the first-order reaction rate constant. These findings greatly simplify the use of natural stable isotope changes in CH_4 to assess the extent that CH_4 is oxidized in seawater following seafloor release.

Plain Language Summary

The aerobic oxidation of methane in seawater is a process that prevents methane produced in the oceanic environment from being emitted to the atmosphere. During this process, isotopic forms of methane are oxidized at slightly different rates leading to changes in the natural methane isotope ratios with the extent of oxidation. While these changes in isotope ratios would seem to be a proxy for the extent of methane oxidation, laboratory-based studies involving pure cultures have shown that these isotope ratio changes vary as a microbial population blooms in response to an increase in substrates. This study systematically measured the stable isotope changes that are associated with aerobic methane oxidation in recently collected seawater collected from regions of active seafloor methane release along the U.S. Atlantic margin and the Gulf of Mexico. Results show that these isotope changes are systematic during methane oxidation, greatly simplifying the use of isotope changes to determine the extent of methane oxidation.

1. Introduction

Kinetic processes, such as microbial oxidation, are known to systematically change the isotopic abundance of various molecules. Typically, the light isotopologue reacts with a slightly faster rate constant than the heavy isotope, leaving the residual reactant isotopically fractionated. For example, studies of oceanic methane (CH_4) have utilized this isotopic fractionation as a proxy for the extent of CH_4 oxidation and have gone so far

as to use it to help determine oxidation rates (e.g., Kessler et al., 2006; Leonte et al., 2017). The oceanic CH_4 reservoir is one of the largest CH_4 reservoirs on Earth, and significant releases of CH_4 from the seafloor into the overlying waters have been documented in the modern ocean (Ruppel and Kessler, 2017). However, the minimal emission of oceanic CH_4 to the atmosphere (Dlugokencky et al., 2011) underscores active CH_4 oxidation in seawater and surface sediments that prevents atmospheric release.

Traditionally, CH_4 oxidation rates are not measured using natural stable isotope changes. Instead, CH_4 oxidation rate measurements are generally conducted by collecting seawater samples in glass vials, inoculating the samples with radioactive or stable isotopically-labeled CH_4 immediately after collection, incubating at in-situ temperatures for a measured time period, and terminating further oxidation with the addition of a toxic agent such as mercuric chloride (e.g. Crespo-Medina et al., 2014; Mendes et al., 2015; Niemann et al., 2015; Pack et al., 2011 and 2015; Valentine et al., 2001; Valentine et al., 2010). The labeled CH_4 allows for tracing the reactant as it is incorporated into product by native methanotrophs. Beyond not maintaining in-situ pressures, a consistent challenge with these techniques is amending the seawater sample while minimizing the disturbance to the natural concentrations of the dissolved gases (Pack et al., 2011), trace metals, and nutrients. Typically, these studies involve multiple samples per rate determination that are often collected in different glass vials and not allowed to incubate in the same reservoir (e.g. Crespo-Medina et al., 2014; Leonte et al., 2017; Mendes et al., 2015; Pack et al., 2011). This creates individual samples - pseudoreplicates - that might proceed at differing oxidation rates due to slight differences in initial microbial populations or in dissolved gases and substrates (e.g., nutrients and trace metals) utilized in the oxidation processes. Further adding to complications, borosilicate glass serum vials that are traditionally used for CH_4 oxidation rate measurements have been shown to leach trace metals (e.g., Fe and Cu) from the borosilicate glass, potentially fertilizing the samples with greater amounts of essential trace metals than occur naturally (Batley and Gardner, 1977; Robertson, 1968). Additionally, certain rubber stoppers used to seal these vials have been shown to have toxic effects on methanotrophs (Niemann et al., 2015).

An alternative to using isotopic labeling techniques for measuring CH_4 oxidation rates is the use of naturally occurring stable isotopes (Leonte et al., 2017). Recent advances in technology enable these measurements to be conducted at sea and in-situ (Chen et al., 2013; Wankel et al., 2012). A strong advantage with this approach is that integrated CH_4 oxidation rates are determined based on the in-situ isotopic conditions without the need to externally incubate samples, which can potentially cause alterations to biological, chemical, temperature, and pressure conditions (Leonte et al., 2017). During CH_4 oxidation, $^{12}CH_4$ is oxidized with a slightly greater rate constant than $^{13}CH_4$, causing the residual CH_4 pool to become relatively enriched in the heavy isotopes over time (Whiticar, 1999). Similarly, the lighter ^{12}C of the oxidized CH_4 has been traced into the products of cellular biomass and CO_2 (Orphan et al., 2001; Radajewski et al., 2000; Summons et al., 1994). This isotopic fractionation process can be used to quantify the extent of CH_4 oxidation if the fractionation factor (α) is known or can be determined

(Leonte et al., 2017). The α is the ratio of the rate constants of the lighter isotope over the heavy isotope assuming first-order kinetics for this oxidation process. Interestingly, culture studies of methane-producing archaea have reported that the fractionation factor changes during microbially mediated reactions as the microbial population grows (Botz et al., 1996; Penning et al., 2005; Valentine et al., 2004), potentially complicating the use of natural isotopes and isotopic fractionation to quantify CH_4 oxidation in natural environments that are not in steady-state conditions. This complication would be especially noticeable in environments where the dissolved CH_4 concentration rapidly increased in a parcel of water, such as a seep field or hydrocarbon spill (e.g. Kessler et al., 2011; Leonte et al., 2017), resulting in a significant bloom of the CH_4-oxidizing population. This proxy would only be quantitative during a microbial bloom/oxidation event once CH_4 oxidation isotopic fractionation factors stabilized. Thus, this study was motivated by the need to thoroughly quantify the changes in stable isotope kinetics as a population of methanotrophic bacteria grew to oxidize an enhanced CH_4 input.

Here we conducted mesocosm experiments with CH_4-laden seawater measuring isotopic changes over time during CH_4 oxidation events. The goal of this investigation was to determine fundamental stable isotopic fractionation parameters associated with aerobic CH_4 oxidation. To assess potential regional variabilities in CH_4 oxidation kinetics, seawater was collected in two different locations where CH_4 bubbles were seeping from the seafloor: a) Hudson Canyon off the coast of New York and New Jersey near the upper limit of CH_4 hydrate stability and b) the deep Gulf of Mexico near waters once impacted by the Deepwater Horizon blowout. The results of these studies can be used to help quantify the extent and rates of CH_4 oxidation based on natural changes of CH_4 isotopes.

2. Materials and Methods

All information relating to the seawater sample collection, incubation, and analysis can be found in the companion paper (Chan et al., submitted). To briefly summarize, waters directly influenced by, or adjacent to, known CH_4 seep activity were chosen to examine CH_4 oxidation kinetics. The first research expedition was aboard the R/V *Endeavor* along the North Atlantic Bight from 7-12 July 2014. The recently discovered CH_4 seeps off the coast of New York and New Jersey in Hudson Canyon (HC) (Rona et al., 2015; Skarke et al., 2014; Weinstein et al., 2016) provided an appropriate site for these experiments. Water samples were collected both inside the seep field (39° 32.705'N, 72° 24.259'W) as well as outside of HC in waters not directly impacted by CH_4 seeps (39° 17.236'N 72° 12.080'W), as determined by the presence or absence of acoustically detected bubbles (Leonte et al., 2017; Weinstein et al., 2016). These water samples were collected via Niskin bottles that were pre-cleaned for trace-metal analyses. A measured amount (150 ± 1.5 mL) of isotopically standardized CH_4 ($\delta^{13}C\text{-}CH_4$ = -20 ‰; Kessler and Reeburgh (2005)) was systemically added to each sample using a mass flow controller and gas filter apparatus to increase dissolved CH_4 concentrations to ca. 300 μM CH_4. Therefore, these mesocosm incubations using waters collected from the HC region began with similar values for dissolved CH_4 concentration and $\delta^{13}C\text{-}CH_4$.

A second research expedition was conducted from 9-20 April 2015 aboard the E/V *Nautilus* at the Sleeping Dragon seep field site (MC118) in the Gulf of Mexico. MC118 is 17 km from the Deepwater Horizon (DWH) wellhead and provided physical-chemical conditions similar to what may have been experienced during the DWH hydrocarbon spill in 2010. The Suspended-Particle Rosette (SUPR) sampler (Breier et al., 2009) was mounted to the ROV *Hercules* and was used for the high precision collection of water that was visibly impacted by CH_4 bubbles. This sampling strategy enabled water to be collected that contained naturally high concentrations of dissolved CH_4, so no additional CH_4 was added. The results obtained from HC and MC118 were analyzed to determine regional similarities and variabilities in CH_4 oxidation kinetics.

Full details of the sample collection, analysis, and calibration procedures can be found in the companion paper (Chan et al., submitted). In addition, the specific details and validation tests for the system used to incubate and analyze these mesocosms are presented in Chan et al. (2016). Finally, all data and descriptions of the analyses from these experiments are available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) (Kessler and Chan, 2017).

3. Results and Discussion

3.1. General Stable Isotope Changes

Every incubation experiment was examined to determine whether clear signs of aerobic CH_4 oxidation had occurred by comparing changes in dissolved gas concentrations, isotope measurements, microbial community, cell densities, and micro- and macro-nutrients (see companion paper for complimentary analyses). Six of the ten mesocosms with waters collected inside and adjacent to HC exhibited CH_4 oxidation (Figures 1A-B and S1). Four of the ten mesocosms collected with waters at MC118 displayed clear characteristics of CH_4 oxidation (Figures 1C-D and S3-S4). While we do not have clear evidence to explain this observation, we offer two possibilities. First, the mesocosms may have grown successfully but a partial blockage in the water sampling tubes may have prevented accurate measurements. Second, the process of removing the samples from their deep ocean environment may have harmed the indigenous population.

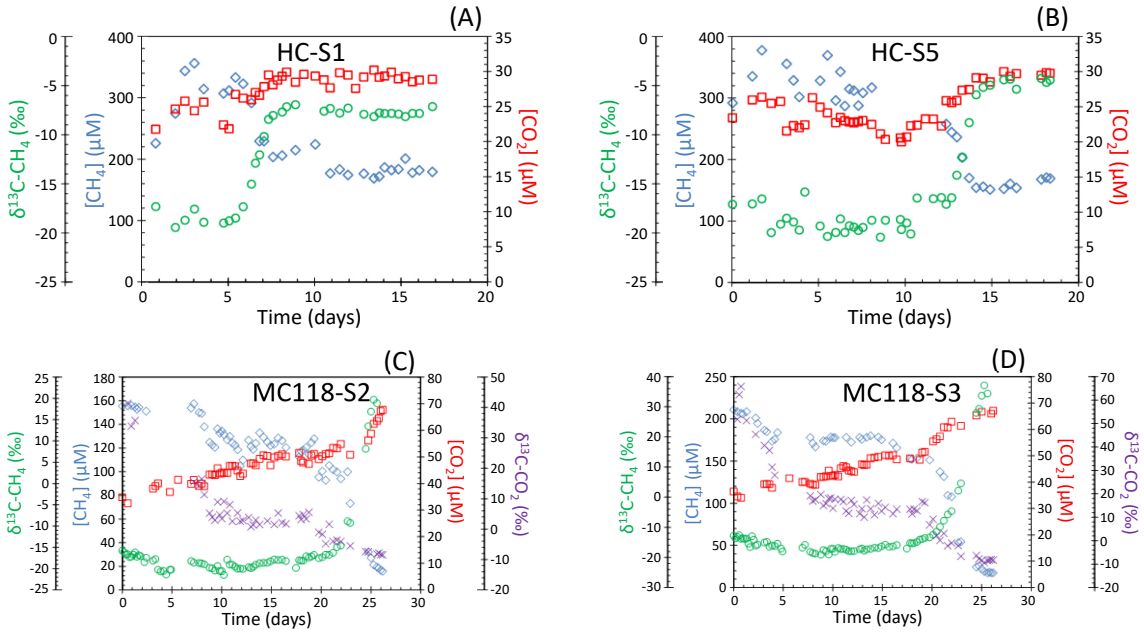

Figure 1. Dissolved concentrations of CH_4 (blue diamonds), CO_2 (red squares), $\delta^{13}C$-CH_4 (green circles) (Chan et al., submitted), and $\delta^{13}C$-CO_2 (purple ×) over the course of the incubations. (A) HC-S1 (on seep), (B) HC-S5 (off seep), (C) MC118-S2 (on seep) and (D) MC118-S3 (on seep). All data in these figures is available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) (Kessler and Chan, 2017).

Along with the measurement of the changes in chemical concentrations and microbial populations (see companion paper), $\delta^{13}C$-CH_4 and $\delta^{13}C$-CO_2 were measured using real-time monitoring to assess microbial isotopic fractionation of the substrate and product. Since the HC samples were equilibrated with standardized CH_4, these incubations began close to the $\delta^{13}C$-CH_4 standardized value of -20 ‰ (Chan et al., submitted; Kessler and Reeburgh, 2005). The HC mesocosms showed an average change in $\delta^{13}C$-CH_4 of 11 ± 1 ‰ to more positive (heavier) values (Figures 1 and S1, Table S1). The measurement of $\delta^{13}C$-CO_2 was unsuccessful for the HC mesocosms due to a manifold failure; however, the system was redesigned for the MC118 experiments producing usable results.

The starting $\delta^{13}C$-CH_4 for MC118 varied based on the natural input of dissolved CH_4 supplied by the Sleeping Dragon seep. The average starting $\delta^{13}C$-CH_4 was approximately -28 ± 11 ‰ and for those samples exhibiting oxidation, the average increase in $\delta^{13}C$-CH_4 was 37 ± 15 ‰ (Figures 1, S3, and S4, and Table S1). Additionally, CO_2 became more depleted in ^{13}C over time with an average change of -10 ± 10 ‰ (Figure S4 and Table S1). These isotopic shifts ($\delta^{13}C$-CH_4 to heavier values and $\delta^{13}C$-CO_2 to lighter values) throughout these incubations suggest that CH_4 oxidation is occurring and transferring carbon from the dissolved CH_4 pool to the dissolved CO_2 pool.

3.2. Fractionation Factor (α) for Aerobic CH_4 Oxidation

The isotopic fractionation factor (α) is a constant describing the extent that isotopes of one compound change during a kinetic process, fundamentally defined in this experiment as the ratio of the first-order rate constants for the oxidation of $^{12}CH_4$ over $^{13}CH_4$ (Eq. 1).

$$\alpha = {}^{k_{12C}}/_{k_{13C}} \qquad \text{(Eq. 1)}$$

where k is the first-order rate constant with units of day^{-1} such that,

$$\text{Rate} = k[CH_4] \qquad \text{(Eq. 2)}$$

and $[CH_4]$ is dissolved CH_4 concentration.

Since the mesocosm incubations are closed-system experiments, not allowing for the addition or loss of reactants or products to the outside environment, isotopic fractionation can be modeled with the Rayleigh equation (Bigeleisen and Wolfsberg, 1958). This allows for calculating α using a method previously outlined in Leonte et al. (2017). This method linearizes the Rayleigh equation and determines α from the slope of the linearized data (Eq. 3).

$$\ln[CH_4] = \frac{\alpha}{1-\alpha} * \ln[\delta R + 1000] - \frac{\alpha}{1-\alpha} * \ln[\delta R_0 + 1000] + \ln[CH_{4,0}] \qquad \text{(Eq. 3)}$$

Here, $[CH_4]$ is the dissolved CH_4 concentration, $[CH_{4,0}]$ is the dissolved CH_4 concentration at the start of the reaction, δR is δ^{13}C-CH_4, and δR_0 is δ^{13}C-CH_4 at the start of the reaction. The slope of $\ln[CH_4]$ vs. $\ln[\delta R + 1000]$ is α/(1-α), which can be rearranged to determine α (Leonte et al., 2017). The geometric mean regression is used here when determining the linear regression between $\ln[CH_4]$ and $\ln[\delta R + 1000]$ because it takes into account uncertainty in both variables.

The average α for the mesocosms collected in the HC over the seep field was 1.023 ± 0.003. The MC118 mesocosms produced an average α of 1.022 ± 0.003, which is statistically similar to the values obtained directly in the seep field in HC. However, α was different for the experiments utilizing water that was not directly impacted by CH_4 bubbles released from a seep field, displaying an average α = 1.04 ± 0.01. In addition to displaying a higher average α, the HC mesocosms using water collected outside the seep field also displayed lower average rate constants (see companion paper), supporting the conclusion that slower rates of CH_4 oxidation produce larger fractionation factors (Table 1; Figure 2). Viewed differently, larger oxidation rate constants cause lower degrees of isotopic fraction, a conclusion which can be used to predict the largest oxidation rate constant which would ultimately produce no isotopic fractionation (i.e., α = 1). To constrain this endmember, we calculated a linear regression between k and α again using

246 a geometric mean regression since it takes into account uncertainty in both variables
247 (Figure 2). This regression predicts a maximum rate constant (k_{max}) of 0.44 day^{-1} to
248 produce no isotopic fractionation. However, this conclusion is rather uncertain (k_{max} =
249 0.44 ± 3.9 day^{-1}) when the uncertainties in both the slope and y-intercept of the geometric
250 mean regression ($k = (-9.3 \pm 2.7)\alpha + (9.8 \pm 2.8)$) are propagated. Nonetheless, it is
251 interesting to note the similarities between this maximum rate constant which would
252 produce no isotopic fraction and the rate constants measured during and after the DWH
253 blowout (Figure 2, and Figure 1 in Chan et al., submitted).

254 **Table 1.** The characteristics for isotopic kinetics determined in Hudson Canyon (HC) and
255 MC118. The units for the first-order oxidation rate constants (k) is day^{-1} and isotopic
256 fractionation factors (α) in unitless ($day^{-1/}$ day^{-1}).
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Sample	Location	k (day^{-1})	α
HC-S1	On seep	0.25 ± 0.03	1.021 ± 0.002
HC-S2	On seep	0.18 ± 0.04	1.025 ± 0.005
Average	On seep	0.22 ± 0.05	1.023 ± 0.003
HC-S3	Off seep	0.054 ± 0.004	1.054 ± 0.007
HC-S4	Off seep	0.12 ± 0.01	1.032 ± 0.002
HC-S5	Off seep	0.24 ± 0.03	1.023 ± 0.001
HC-S6	Off seep	0.061 ± 0.003	1.034 ± 0.003
Average:	Off seep	0.12 ± 0.09	1.04 ± 0.01
MC118-S1	On seep	0.107 ± 0.005	1.025 ± 0.001
MC118-S2	On seep	0.26 ± 0.04	1.024 ± 0.002
MC118-S3	On seep	0.36 ± 0.04	1.024 ± 0.001
MC118-S4	On seep	0.20 ± 0.02	1.016 ± 0.001
Average:	On seep	0.2 ± 0.1	1.022 ± 0.004

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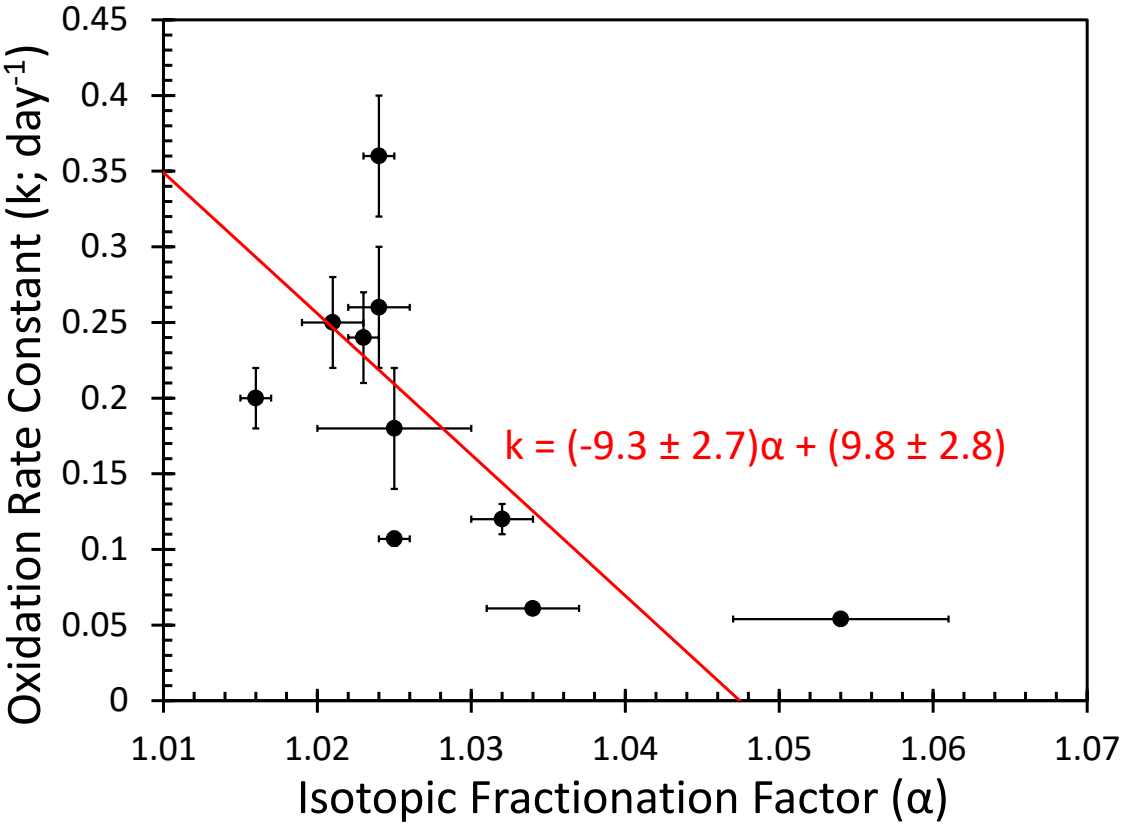

Figure 2. The first-order rate constant (k) for aerobic CH_4 oxidation as a function of isotopic fractionation factor (α) for $\delta^{13}C$-CH_4. The linear regression was determined using the Geometric Mean to consider the uncertainties in both the rate constant and isotopic fractionation factor.

3.3. Isotopic Fraction with the Stage of Microbial Growth

Previous studies have collected data from laboratory cultures of methanogens suggesting that isotopic fractionation factors change with the stage of microbial growth (Botz et al., 1996; Penning et al., 2005; Valentine et al., 2004). If applicable to aerobic methanotrophs, the use of natural stable isotopes to determine the extent of CH_4 oxidation (e.g., Leonte et al., 2017) would be significantly more complicated, if not impossible. Not only would the fractionation factor need to be known, but also how it changes with the stage of microbial growth and the stage of microbial growth in the natural environment at the time of sampling.

Here we test if the isotopic fractionation factor changed throughout rapid CH_4 oxidation. To do so, we isolated the data between the start and conclusion of rapid CH_4 oxidation (Kessler and Chan, 2017) and fit the data with the closed-system Rayleigh isotope fractionation model (Eq. 4), as was described previously (Leonte et al., 2017).

$$\delta R = (\delta R_0 + 1000)(1 - f)^{1/\alpha - 1} - 1000 \quad \text{(Eq. 4)}$$

The variables of δR, δR_0, and α are as defined above, while f is the fraction of CH_4 that is oxidized. More specifically, $f = 1 - C/C_0$, where C is the measured [CH_4] and C_0 is the average concentration of dissolved CH_4 at the start of rapid CH_4 oxidation (Leonte et al., 2017).

While the previous experiments that displayed a change in isotopic fractionation factor with the stage of microbial growth involved open-system pure cultures of methanogens (Botz et al., 1996; Penning et al., 2005), our data from these closed-system mesocosm experiments investigating methanotrophy could be modeled with a constant isotopic fractionation factor after rapid CH_4 oxidation initiated (Figures 3, S2, and S5), despite large changes in microbial biomass (see companion paper). This result greatly simplifies the use of natural stable isotope changes in seawater to determine the extent of aerobic oxidation since only one fractionation factor is necessary to estimate the extent of CH_4 oxidation (e.g. Leonte et al., 2017).

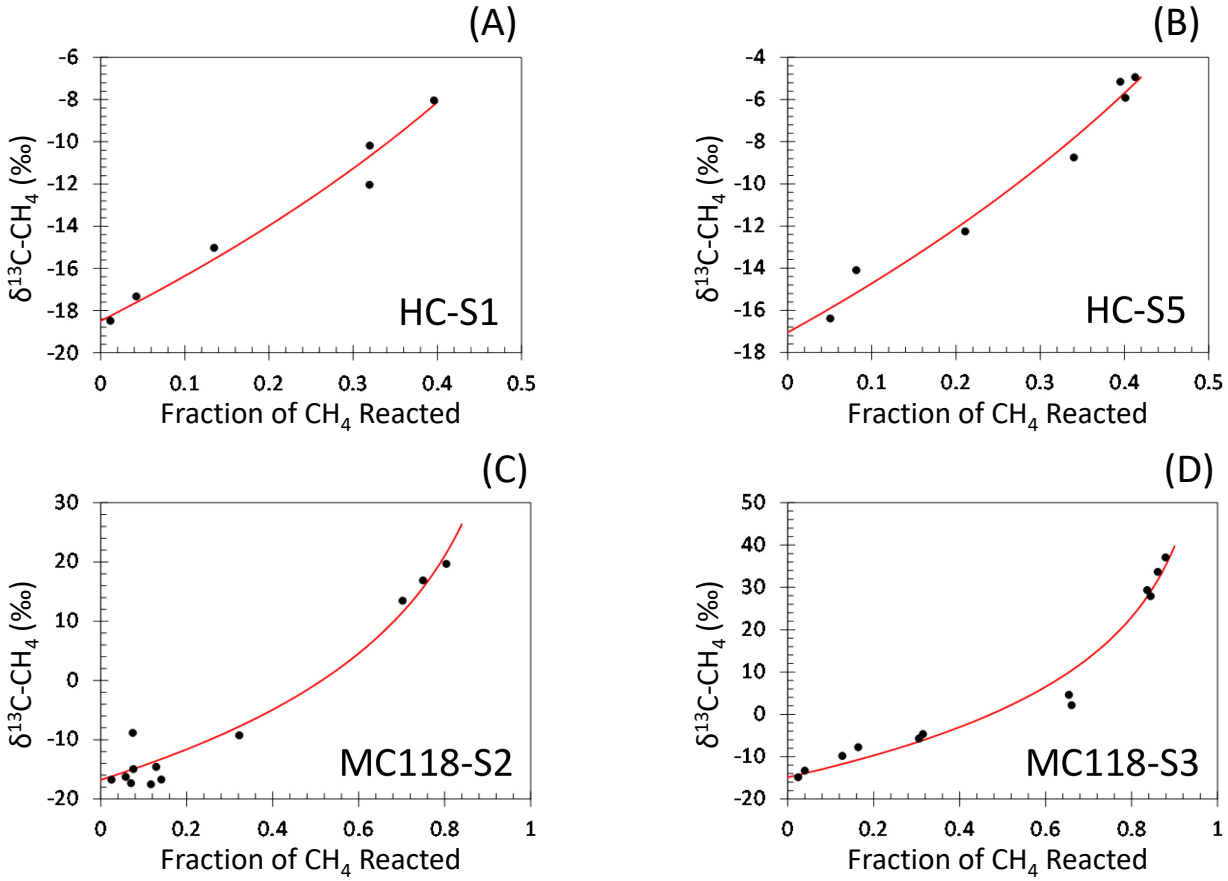

Figure 3. Values of δ^{13}C-CH_4 plotted as a function of the fraction of CH_4 reacted in these mesocosm experiments. The model (red curve) fits the measurements (black dots) when a constant isotopic fractionation is considered (Table 1). The model incorporates the closed-system Rayleigh approach (Eq. 4) and an average CH_4 concentration at the start of rapid CH_4 oxidation, as described previously (Leonte et al., 2017).

4. Conclusions

Measurements of natural δ^{13}C-CH_4 in seawater are a powerful tool to assess the extent of aerobic oxidation. Following seafloor release and dissolution into the overlying waters, CH_4 can either be oxidized by indigenous microorganisms or diluted prior to atmospheric emission. While both oxidation and dilution decrease the initial dissolved CH_4 concentration, only oxidation systematically changes the natural δ^{13}C-CH_4. Here, we investigated the stable isotope kinetics of aerobic CH_4 oxidation using mesocosm incubations of seawater collected in two seep fields, one in the North Atlantic Bight in and near Hudson Canyon and the other in the Gulf of Mexico. The results produced from these experiments led to three conclusions regarding the fundamental isotope kinetics of aerobic CH_4 oxidation. First, the isotope data is best modeled with the Rayleigh model, an isotope model for a closed-system following first-order reaction kinetics. Second, the fractionation factor produced was correlated with the overall reaction rate constant. Reactions with larger rate constants had smaller fractionation factors and vice versa. While the isotopic fractionation factors were different between on-seep and off-seep waters at HC, the

316 isotopic fractionation factors were similar when using waters directly impacted by CH_4 seeps,
317 regardless of oceanic location. Third, despite a large increase in microbial biomass during these
318 oxidation experiments and previous reports concluding that isotope fractionation factors changed
319 with the stage of microbial growth, the results here indicate that the fractionation factor remained
320 constant throughout these oxidation events. These fundamental results are encouraging, as they
321 suggest that the extent of CH_4 oxidation can be determined using traditional isotopic
322 fractionation equations without regard to the stage of microbial growth so long as the
323 fractionation factor can be determined for that specific environment.

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