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# Carbon and hydrogen isotope fractionation associated with the aerobic microbial oxidation of methane, ethane, propane and butane

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**Abstract**

Carbon isotope fractionation factors associated with the aerobic consumption of methane (C1), ethane (C2), propane (C3), and *n*-butane (C4) were determined from incubations of marine sediment collected from the Coal Oil Point hydrocarbon seep field, located offshore Santa Barbara, CA. Hydrogen isotope fractionation factors for C1, C2 and C3 were determined concurrently. Fresh sediment samples from two seep areas were each slurried with sea water and treated with C1, C2, C3 or C4, or with mixtures of all four gases. Triplicate samples were incubated aerobically at 15 °C, and the stable isotope composition and headspace levels of C1–C4 were monitored over the course of the experiment. Oxidation was observed for all C1–C4 gases, with an apparent preference for C3 and C4 over C1 and C2 in the mixed-gas treatments. Fractionation factors were calculated using a Rayleigh model by comparing the $\delta^{13}C$ and $\delta D$ of the residual C1–C4 gases to their headspace levels. Carbon isotope fractionation factors (reported in $\varepsilon$ or $(\alpha - 1) \times 1000$ notation) were consistent between seep areas and were $-26.5‰ \pm 3.9$ for C1, $-8.0‰ \pm 1.7$ for C2, $-4.8‰ \pm 0.9$ for C3 and $-2.9‰ \pm 0.9$ for C4. Fractionation factors determined from mixed gas incubations were similar to those determined from individual gas incubations, though greater variability was observed during C1 consumption. In the case of C1 and C3 consumption, carbon isotope fractionation appears to decrease as substrate becomes limiting. Hydrogen isotope fractionation factors determined from the two seep areas differed for C1 oxidation but were similar for C2 and C3. Hydrogen isotope fractionation factors ranged from $-319.9‰$ to $-156.4‰$ for C1 incubations, and averaged $-61.9‰ \pm 8.3$ for C2 incubations and $-15.1‰ \pm 1.9$ for C3 incubations. The fractionation factors presented here may be applied to estimate the extent of C1–C4 oxidation in natural gas samples, and should prove useful in further studying the microbial oxidation of these compounds in the natural environment.


## 1. Introduction

Hydrocarbon gases including methane ($CH_4$; C1), ethane ($C_2H_6$; C2), propane ($C_3H_8$, C3), and *n*-butane (*n*-$C_4H_{10}$, *n*-C4, also C4 hereafter) are produced naturally in deep subsurface environments and represent the major components of natural gas. The hydrocarbons composing natural gas are produced primarily from the decomposition of organic material. When decomposition is biologically mediated C1 is the dominant product (biogenic gas), and higher hydrocarbons are present only at trace levels (Schoell, 1988). When gas production is due to thermal decomposition (thermogenic gas) C2–C4 are more abundant, frequently comprising 10% or more of the gas. Natural gas is widely distributed in the subsurface due to its high mobility and is mined from the subsurface in areas where geologic traps have led to gas accumulation (Hunt, 1996). The conventional or recoverable global reservoir of natural gas is estimated at 100,000 Teragrams ($Tg = 10^{12}$ g) and is currently being used at a rate of 1500 Tg $yr^{-1}$ (Energy Information Administration, 2004). Global unconventional gas resources in basement rock, shales, coal and hydrates are not well quantified but are estimated to greatly exceed conventional resources (Hunt, 1996). C1 is the principle

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component of natural gas, and has received significantly more attention than C2–C4, particularly because of its importance to energy, climate change and atmospheric chemistry (Cicerone and Oremland, 1988; Fung et al., 1991; Prather and Ehhalt, 2001).

The atmospheric biogeochemical cycles of C2–C4 hydrocarbons are similar to that of C1 in that emissions are primarily terrestrial and loss is primarily atmospheric. As with C1, the atmospheric levels of C2–C4 hydrocarbons are elevated in the Northern Hemisphere and display annual cycles related to their removal by OH radicals (Singh and Zimmerman, 1992; Clarkson et al., 1997). However, there are several important differences. The mixing ratios of C2–C4 are as much as three orders of magnitude lower than C1 in unpolluted air (Kanakidou et al., 1991), and typical atmospheric residence times in the tropics are 20 days for C2, 5.3 days for C3 and 2.1 days for *n*-C4 (Singh and Zimmerman, 1992), compared to 8.4 years for C1 (Prather and Ehhalt, 2001). Sources of atmospheric C2 include biomass burning (6.4 Tg $yr^{-1}$; Greenberg and Zimmerman, 1984), soils (0.4 Tg $yr^{-1}$; Rudolph, 1995), vegetation (flux unknown; Warneck, 1988), and oceans (0.2–0.5 Tg $yr^{-1}$; Rudolph, 1995). However, two major sources of atmospheric C1, natural wetlands and rice cultivation, are not significant sources of C2, C3 or C4 (Rudolph, 1995; Matthews, 2000; Prather and Ehhalt, 2001). Leakage of C2 from subsurface reservoirs due to natural gas loss during gas and oil drilling, coal mining, gas venting, gas transmission, and transportation is substantial and has been estimated at 2.6–11 Tg $yr^{-1}$ with a best estimate of 6 Tg $yr^{-1}$ (Rudolph, 1995). Sources of C3 and C4 alkanes are not as well constrained but generally correspond with those of C2 and include biomass burning (Clarkson et al., 1997; Scholes et al., 2003), oceans (Saito et al., 2000), and natural gas leakage (Wang et al., 2000). The amount of radiocarbon-free C1 in the atmosphere is higher than the amount of C1 estimated from global anthropogenic natural gas loss (Lacroix, 1993; Etiope and Klusman, 2002), raising the possibility of significant geologic emission of C1–C4 hydrocarbons into the atmosphere through gas permeable faults and fractured rock. Alternatively, release of C1 from older peat deposits may also account for this observation (Zimov et al., 1997). Natural and anthropogenic emissions of C2–C4 contribute to atmospheric pollution at the regional level as a result of photochemical reactions involving the hydroxyl radical and nitrogen oxides (Singh and Zimmerman, 1992).

Microbes are known to affect the distributions of hydrocarbons, primarily through their capacity to consume hydrocarbons in the presence of a suitable oxidant (Atlas, 1995; Hanson and Hanson, 1996; Widdel and Rabus, 2001). The microbial consumption of C1 is referred to as methanotrophy. This process has been the subject of numerous investigations due to its importance in the C1 cycle and because of the unique biochemistry involved (Cicerone and Oremland, 1988; Reeburgh et al., 1993; Hanson and Hanson, 1996). C1 oxidation occurs in both oxic and anoxic environments; the focus of this study is on aerobic oxidation. The enzymes responsible for this activity are the two primary forms of methane monooxygenase (MMO): the particulate (pMMO) and soluble (sMMO) forms (Hanson and Hanson, 1996). Both MMO enzymes are able to activate a range of multi-carbon substrates including short chain alkanes and alkenes, and sMMO is also known to activate ethers, alicyclics, aromatics and heterocyclics (Colby et al., 1977). Some aerobic methanotrophs have recently been shown to grow facultatively with concurrent consumption of both C1 and multi-carbon organic substrates. Dedysh et al. (2005) report that the addition of acetate to methanotrophic cultures may serve as a long term stimulus for C1 oxidation. In this case, the addition of acetate also lowered the concentration threshold for C1 oxidation. However, the effect of alternative substrates on the uptake of C1 is not well established, especially with regard to the presence of higher hydrocarbons in environmental systems.

The microbial consumption of C2–C4 hydrocarbons has been investigated previously, though not to the same extent as methanotrophy. C2–C4 alkane oxidation seems to be limited to a group of gram-positive organisms in the *Corynebacterium–Nocardia–Mycobacterium–Rhodococcus* group (Ashraf et al., 1994), along with some gram-negative *Pseudomonas* species (Takahashi, 1980; Hamamura et al., 1999). Some C3 consuming bacteria utilize monooxygenase enzymes to oxidize alkanes to alcohols by attacking the subterminal and terminal carbon positions (Perry, 1980; Stephens and Dalton, 1986; Ashraf et al., 1994). A terminal oxidation pathway was determined for the C4 oxidizing *Pseudomonas butanovora* (Arp, 1999). Alkane monoxygenases also demonstrate broad specificity; it has been shown that C2–C4 grown bacteria can degrade chlorinated hydrocarbons (Hamamura et al., 1997), ammonia (Hamamura et al., 1999; Berthe-Corti and Fetzner, 2002), and ethylene (Hamamura et al., 1999). The C4 monooxygenase of *Nocardioides* sp. strain CF8 was shown to be similar to copper containing monooxygenases such as ammonia monooxygenase (AMO) and particulate methane monooxygenase (pMMO), while other C4 monoxygenases found in *P. butanovora* contained functional heme groups (Hamamura and Arp, 2000). *Pseudomonas putida* was recently identified as being capable of C3 and C4 oxidation using an alkane hydroxylase enzyme (Johnson and Hyman, 2006).

The distribution of stable isotopes ($\delta^{13}C$ and $\delta D$) in hydrocarbon gases provides information on the sources and sinks for these gases. For example, the isotopic distributions associated with C1 sources and isotopic fractionation associated with sinks have been used to quantify the global C1 budget (Stevens and Rust, 1982; Stevens, 1988; Quay et al., 1991, 1999; Lassey et al., 1993; Gupta et al., 1996; Tyler et al., 1999; Miller et al., 2002) and are used routinely for regional and process-oriented investigations (Reeburgh et al., 1991; Bilek et al., 1999; Valentine et al., 2001; Grant and Whiticar, 2002; von Fischer and

Hedin, 2002; Heeschen et al., 2004; Kessler et al., 2005; Heeschen et al., 2005). In deep subsurface hydrocarbon reservoirs, the production of $^{13}C$ depleted, biogenic C1 is a major process mediated by anaerobic methanogens, most likely involving syntrophic bacteria that also utilize higher hydrocarbons (Zengler et al., 1999). The isotopic composition in C2–C4 hydrocarbon gases is routinely used to infer the conditions at which the gas was formed (James, 1983; Whiticar, 1990). James and Burns (1984) noted significant carbon isotopic enrichments in C3 and C4 from deep subsurface hydrocarbon reservoirs, which they used to infer microbial consumption of these gases. Preference for C3 degradation followed by C4 and then C2 has been noted as well (Boreham et al., 2001), with particularly high levels of biodegradation in lower temperature (<60 °C) reservoirs (Wenger et al., 2002). Boreham et al. (2004) found biodegradation of C2–C4 hydrocarbons in Australian natural gases resulting in hydrogen isotope enrichments of over an order of magnitude greater than corresponding carbon isotope enrichments. Here, the C2 and C3 hydrocarbons showed over 200‰ enrichments in D compared to their equivalent non-biodegraded natural gases. Such processes in these deep subsurface hydrocarbon reservoirs are more likely to involve anaerobic microorganisms (Head et al., 2003). Formolo et al. (2004) used the isotopic composition of carbonate-associated gas-hydrates in the Gulf of Mexico to argue for the importance of C2+ hydrocarbons to carbon cycling around thermogenic gas hydrates. Few other investigations have used the isotopic composition of C2–C4 to investigate the biogeochemical cycling of these gases, and no fractionation factors are currently available for their microbial consumption.

In this work, we investigate the aerobic microbial consumption of C1–C4 hydrocarbons in marine sediment samples from natural seeps of thermogenic gas. Specifically, we seek to determine whether C1–C4 oxidation occurs in simulated seep environments, how the C1–C4 composition impacts patterns of gas consumption, the magnitude of carbon isotope fractionation during microbial oxidation of C1–C4, and the magnitude of hydrogen isotope fractionation associated with the microbial oxidation of C1–C3.

## 2. Methods

### 2.1. Sediment source and collection

The sediment samples used in this study were collected from the surface sediment layer at two distinct seep environments located offshore Santa Barbara, CA. The first site is informally referred to as Shane Seep (34°N, 24.370′; 119°W, 53.428′) and is located at 21 m water depth in the Coal Oil Point seep field (Allen et al., 1970). The composition of gas emitted from the seafloor at Shane Seep is 79.7% C1, 15.2% $CO_2$, 0.87% C2, 0.72% C3, and 0.23% C4. Shane Seep is estimated to emit approximately 1.9 tons C1 per day to the atmosphere (Washburn et al., 2005). The second site is referred to as Brian Seep (34°N, 24.109′; 119°W, 49.917′) and is located at 10 m water depth about 7 km removed from the center of the Coal Oil Point seep field. Gas flux from this seep area has not been quantified, but the estimated flux is 2–3 orders of magnitude lower than from Shane Seep. The composition of gas emitted from the seafloor at Brian Seep is 91.2% C1, 7.5% $CO_2$, 1.1% C2, 0.2% C3, with less than 0.05% C4. Both locations represent minor seeps in the Coal Oil Point seep field, which is estimated to emit $80 \pm 12$ tons of C1 $day^{-1}$ and $26 \pm 12$ tons of gaseous C2+ hydrocarbons $day^{-1}$ (Hornafius et al., 1999). The sediment at Brian Seep is composed primarily of sand, and little tar is apparent in the sediments, whereas sediment from Shane Seep is silty and contains greater amounts of residual tar. Samples were collected by scuba diver, using a hand shovel to scrape the top 2–3 cm of sediment into a sealable 1 L plastic container. Samples were collected January 19th, 2005 at Brian Seep and January 25th, 2005 at Shane Seep, and were returned to the lab within 1–2 h.

### 2.2. Sample processing

Once sediment samples were returned to the laboratory, sterile seawater was added to the sediment in a 1:1 ratio (volume to volume) and the resulting slurry was homogenized. Samples were prepared in triplicate as follows. Fifty milliliters of the sediment slurry was added to a 150 mL serum bottle, which was crimp-sealed with a gas-tight butyl rubber stopper. The headspace in the serum bottle was subsequently purged with ultra-high purity (UHP) $N_2$ gas for 5 min at approximately 150 mL $min^{-1}$. Each sample was then amended with the addition of oxygen and hydrocarbon gases to the headspace. The proportions of hydrocarbon and oxygen were chosen based on the stoichiometry for complete hydrocarbon oxidation, such that the hydrocarbon gas would be the limiting reactant but that the initial $O_2$ would not exceed 20% of the headspace gas. The expected reaction stoichiometries for the complete aerobic oxidation of C1–C4 hydrocarbons, exclusive of biomass generation, are as follows:

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O \quad (1)$$

$$C_2H_6 + 3.5O_2 \rightarrow 2CO_2 + 3H_2O \quad (2)$$

$$C_3H_8 + 5O_2 \rightarrow 3CO_2 + 4H_2O \quad (3)$$

$$C_4H_{10} + 6.5O_2 \rightarrow 4CO_2 + 5H_2O \quad (4)$$

After these additions a small quantity of UHP $N_2$ was added as an overpressure such that subsequent sampling did not draw a vacuum. Two types of incubations were performed: those with only one hydrocarbon gas and those with a mix of C1–C4. The average (±SD) initial mixing ratios for C1–C4 in the individual gas incubations were $7.0\% \pm 0.8$ for C1 treatments, $3.8\% \pm 0.3$ for C2 treatments, $2.1\% \pm 0.4$ for C3 treatments, and $0.9\% \pm 0.3$ for C4 treatments. The average (±SD) initial mixing ratios for the mixed gas incubations were $0.91\% \pm 0.03$ for C1, $0.87\% \pm 0.03$ for C2, $0.71\% \pm 0.1$ for C3, and $0.43\% \pm 0.05$ for C4.

Incubations for all four gases were performed in triplicate for sediment from each seep, with duplicate killed controls and blanks, totaling 19 treatments per seep. Killed controls were heat sterilized twice (121 °C for 15 min) and contained headspace gases in similar proportion to the mixed gas incubations. Blank treatments consisted of sediment slurry without any exogenous hydrocarbon, and served as a control against possible hydrocarbon production. All experimental and control treatments were incubated at 15 °C in the dark on an orbital shaker set at 35 rpm.

### 2.3. Mixing ratio and stable isotope analyses

The gas headspace of each experimental treatment was sampled at intervals of 5–7 days, and samples were stored for later analysis of gas composition as well as hydrocarbon $\delta^{13}C$ and $\delta D$. Gas samples were removed by syringe and transferred to 5 mL crimp-sealed serum bottles. In order to avoid diluting the sample during transfer, the storage bottles were filled completely with water, which was then displaced with the incoming gas sample using a dual syringe displacement technique. Once the gas samples were collected, they were stored in the dark at room temperature prior to analysis.

Hydrocarbon mixing ratios were determined by gas chromatography (GC), using a Shimadzu GC-14A equipped with a flame ionization detector (FID). C1–C4 compounds were resolved on the GC–FID with isothermal 60 °C runs using $N_2$ as the carrier gas (20 mL $min^{-1}$ flow rate) through a $12' \times \frac{1}{8}''$ packed column (*n*-octane on Res-Sil C). Headspace levels are reported as % of headspace (v/v). Standard error in the mixing ratios is ±3% based on replicate injections of a standard. Samples were removed by syringe from the storage bottles using a dual syringe technique by displacing the gas headspace with water and subsequently passing 0.7 mL through a 100 μL sample loop. $O_2$ levels were monitored using a 3000A microGC (Agilent) equipped with a thermal conductivity detector.

Stable carbon isotope ratios ($^{13}C/^{12}C$) of C1–C4 hydrocarbons were measured for selected time points by continuous flow isotope ratio mass spectrometry (IRMS). This was achieved using a Delta Plus XP IRMS coupled with a Trace Gas chromatograph via a GC/Combustion III interface (system components all from Thermo Finnigan, now ThermoElectron), at the UCSB Marine Science Institute's Analytical Laboratory. A 30 m × 0.32 mm ID CarbonPlot capillary column (Agilent Technologies) was used with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The size of the injected gas sample was 0.05–1.0 mL, depending on the mixing ratio. Individual hydrocarbons were resolved using an isothermal temperature setting: 60 °C for C1, 110 °C for C2, 160 °C for C3 and 210 °C for C4. For samples with C1–C4 hydrocarbon mixtures, a temperature program starting at 60 °C for two minutes and ramping to 250 °C at 20 °C $min^{-1}$ was used. The eluent from the GC passes into the 950 °C Pt/Ni/Cu combustion reactor, in which the organic components are quantitatively converted to $CO_2$ before passing into the mass spectrometer.

$^{13}C/^{12}C$ ratios were measured relative to $CO_2$ reference gas, which was calibrated against NBS19 (NIST SRM #8544), and are expressed in per mil notation (‰) relative to the international standard V-PDB (Pee Dee belemnite carbonate, as established by the International Atomic Energy Agency [IAEA] in Vienna, Austria; Coplen, 1995). The $CO_2$ reference gas was introduced into the mass spectrometer at the beginning and end of each GC run. Overall precision of the GC–IRMS system was ±0.30 per mil for samples with >0.1% hydrocarbon in the sample headspace, based on replicate injections of standard. Precision was lower for samples ranging from 0.01% to 0.05% and are displayed as error bars in Figs 1 and 2. This precision was determined by comparing the amplitude of a given peak with a standard of similar amplitude, and applying the 1$\sigma$ error determined from replicate injections of the latter.

Ratios of D/H were measured in all samples using a Thermo Finnigan model Delta XL-Plus isotope ratio mass spectrometer (IRMS) at UC Irvine. The basic methodology (Hilkert et al., 1999; Rice et al., 2001) is similar to $^{13}C/^{12}C$ analyses, as described above. Differences include a helium carrier flow rate of 2.6 mL $min^{-1}$, the use of an Agilent model 6890 GC, and the use of a pyrolysis reactor (set at 1450 °C) in place of a combustion unit. Multiple aliquots of each sample were injected with a gas-tight syringe (aliquot size ca. 100–300 μL at STP depending on concentration of the hydrocarbon) into the inlet port of the GC. Chromatography was performed isothermally using a 25 m PoraPLOT Q capillary column at 30 °C for both C1 and C2 samples and at 100 °C for C3 samples.

A working reference gas cylinder of UHP $H_2$ from Oxygen Services Co. (Costa Mesa, CA) was assigned a value by comparison with $H_2$ gas purchased from Oztech Gas Co. with an assigned value of −165.3‰ versus v-SMOW. Our working reference gas for the cf-GC/IRMS instrument was assigned the value of −169.4‰ for $H_2$ versus SMOW. Precision of measurement for clean dry calibrated standards of $CH_4$ in air are ±1.1‰ for $\delta D$. Greater error was noted for higher hydrocarbons, and standard deviations (1$\sigma$) for multiple determinations of each sample aliquot are provided as error bars in Fig. 3.

### 2.4. Determination of fractionation factors

Fractionation factors were determined using the following equation, which describes the fractionation that occurs in a single step unidirectional reaction in a closed isotopic system (Mariotti et al., 1981).

$$\varepsilon = \frac{10^3 \times \ln \frac{10^{-3}\delta_f + 1}{10^{-3}\delta_i + 1}}{\ln(1 - f)} \quad (5)$$

where $\delta_i$ is the $\delta^{13}C$ of the introduced hydrocarbon in the initial headspace, $\delta_f$ is the $\delta^{13}C$ of the hydrocarbon in the head-

space at subsequent time points, $\varepsilon$ is the fractionation factor or 'per mil enrichment factor' of the product (the aggregate of biomass and $CO_2$) relative to the hydrocarbon substrate, and $f$ is the fractional yield or fraction of hydrocarbon consumed at the given time. The fractionation factor can be calculated by plotting the numerator of the expression on the right side of Eq. (5) against the denominator ($\ln(1-f)$) for a series of time points and performing a linear regression to solve for the slope (Chu et al., 2004). $\varepsilon$ is most accurate at $\delta$ values close to 0‰ and is related to $\alpha$, another notation commonly used for reporting fractionation factors, by the following equation: (Mariotti et al., 1981)

$$\varepsilon = (\alpha - 1) \times 1000 \quad (6)$$

For ease of use, $\varepsilon$ is used throughout this paper. The isotopic fractionation associated with dissolution of hydrocarbons in water is expected to be small and is not considered in these experiments.

The derivation of Eq. (5) assumes a closed isotopic system. In these experiments gas samples were removed periodically, thus the system was not closed. In order to eliminate the systematic error caused by gas removal, $f$ was calculated as a ratio of mixing ratios and is thus independent of gas removal. However, the use of mixing ratios in place of partial pressure causes an error in $f$ associated with changes in the quantity of bulk gas relative to hydrocarbon substrate. In this case, the consumption of hydrocarbons and oxygen and the production of $CO_2$ all change the quantity of bulk gas and thus the mixing ratio of residual hydrocarbons. The consumption of oxygen and hydrocarbon are partially offset by the production of carbon dioxide. This gives rise to slight systematic error in the reported concentrations. For C1 the error is 2% for $f = 0.1$ and 16% for $f = 0.9$, and decreases with increasing chain length from C1 to C4. This systematic error is incorporated into the results by calculating the error for a given amount of consumption and adjusting the reported fraction of hydrocarbon consumed ($f$), according to the stoichiometry given in Eqs. (1)–(4).

## 3. Results

### 3.1. Hydrocarbon oxidation

Hydrocarbon oxidation was noted in all experimental treatments when $O_2$ was present. No hydrocarbon oxidation was noted in killed controls, and no hydrocarbon production was noted in the un-amended sediment (data not shown). Fig. 1A–D displays the time course changes in headspace levels of C1 (Fig. 1A), C2 (Fig. 1B), C3 (Fig. 1C) and C4 (Fig. 1D) for the incubations involving only one hydrocarbon gas. Some variability is apparent in the rates of C1–C4 oxidation, but the general trend toward consumption is clear. In the case of C4 consumption was nearly quantitative for each replicate and each seep environment. In the case of C1–C3, consumption occurred to a greater extent in the samples collected from Brian Seep, than in those collected from Shane Seep. The abrupt halt in oxidation for C1–C3 in samples collected from Shane Seep corresponds to depletion of $O_2$ in the experimental bottles to levels below our detection limit of ~0.015% (data not shown). $O_2$ depletion was presumably related to the microbial oxidation of native organic material from the sediment, as oxygen was added in stoichiometric excess.

Fig. 2A–D displays the time course changes in headspace C1 (Fig. 2A), C2 (Fig. 2B), C3 (Fig. 2C), and C4 (Fig. 2D) levels for the treatments involving a mixture of C1, C2, C3 and C4 hydrocarbon gases. Results indicate that C3 and C4 are oxidized more rapidly than C1 and C2, and the rate of C1 and C2 oxidation increases after C3 and C4 are consumed. The staggered timing of C1 and C2 oxidation indicates possible preference of C3 and C4 by hydrocarbon oxidizing bacteria. Premature $O_2$ depletion was again noted in the mixed gas experimental treatments originating from Shane Seep.

### 3.2. Fractionation of carbon isotopes

A trend toward carbon isotopic enrichment in the residual hydrocarbon gas is observed in all experimental treatments concurrent with hydrocarbon consumption. The range of observed $\delta^{13}C$ values was from −22.0 to +76.4‰ for C1, −32.1 to +4.2‰ for C2, −33.7 to −14.6‰ for C3, and −30.8 to −18.3‰ for C4. This behavior is typical of a closed isotopic system and can be treated similar to a Rayleigh distillation (Coleman et al., 1981). For the incubations involving only one hydrocarbon gas, Fig. 1E–H displays the change in $\delta^{13}C$ of headspace C1 (Fig. 1E), C2 (Fig. 1F), C3 (Fig. 1G) and C4 (Fig. 1H) as a function ($\ln(1-f)$) of the fraction consumed. Sets of triplicate samples from each of the two seep environments are displayed on each panel. The $\delta^{13}C$ of each hydrocarbon was analyzed only at select intervals, for a total of 3–10 isotopic measurements per bottle (average = 6.0).

Fractionation factors determined from each experimental bottle are provided in Table 1, along with $r^2$ values and overall averages. The initial $\delta^{13}C$ was similar for all six treatments for individual hydrocarbon gases, to within ±5.5‰ for C1, ±0.5‰ for C2, ±0.3‰ for C3 and ±1.4‰ for C4; therefore the graphical representation of the fractionation factors given in Fig. 1 includes all $\delta^{13}C$ values for each hydrocarbon gas. Averages of the carbon isotopic fractionation factors ($\varepsilon_c$) for the individual substrate incubations are −25.8‰ ± 1.6 for C1, −9.3‰ ± 1.1 for C2, −4.9‰ ± 1.1 for C3, and −2.4‰ ± 1.1 for C4. The slope of the aggregate determined from the regression lines in Fig. 1 are slightly different from these values; the difference is 1.1‰ for C1, 0.9‰ for C2, 0.0‰ for C3, and 0.4‰ for C4, and is within the standard error for each gas. In the case of C1 and C3 there is an apparent discontinuity in the fractionation as substrate levels become low (at $f \sim 0.87$ or below −2.0 on the $\ln(1-f)$ scale, equivalent to 0.4% C1 and 0.03% C3 in the headspace). These values are treated separately and are indicated by the dashed lines

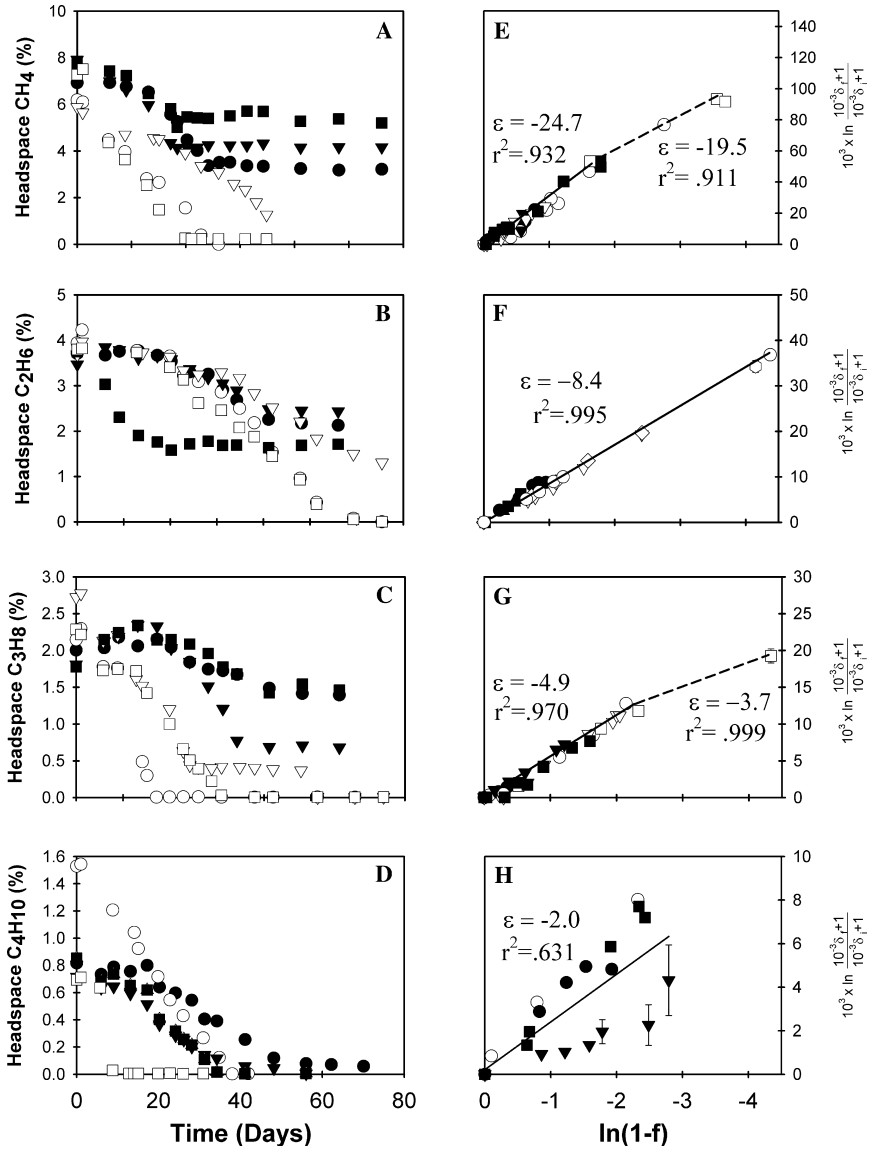


Fig. 1. Incubation of seep sediments with only one hydrocarbon species. (A–D) Display the time course change in C1–C4, respectively, and plots (E–H) display the $\delta^{13}C$ of the corresponding gas as a function ($\ln(1-f)$) of the fraction consumed. Sets of triplicate samples from each of the two seep environments are displayed on each panel. Solid symbols indicate samples from Shane Seep and open symbols indicate samples from Brian Seep. The $\delta^{13}C$ of each hydrocarbon was analyzed only at select intervals. Dashed lines indicate an apparent change in fractionation. Error bars are smaller than the corresponding symbol, except as noted. Sets of triplicate samples from each of the two seep environments are displayed on each panel.

in Fig. 1E and G. The apparent fractionation factors associated with the lower hydrocarbon levels are −19.5 ($r^2 = 0.911$) for C1 and −3.7 for C3 ($r^2 = 0.999$).

Fig. 2E–H displays the $\delta^{13}C$ of headspace C1 (Fig. 1E), C2 (Fig. 1F), C3 (Fig. 1G) and C4 (Fig. 1H) as a function ($\ln(1-f)$) of the fraction consumed—for the incubations involving a mixture of all four hydrocarbon gases. Sets of triplicate samples from each of the two seep environments are displayed on each panel and all treatments are represented on each panel. As with the pure gas treatments, the initial $\delta^{13}C$ was similar for each individual gas in all six treatments, to within ±3.0‰ for C1, ±1.0‰ for C2 and C3, and ±1.6‰ for C4 and linear regressions given in Fig. 2 include all relevant $\delta^{13}C$ values for each hydrocarbon gas individually. The slopes of the regression lines for each experimental treatment are detailed in Table 1, along with $r^2$ values and averages from both seep locations. Averages of the carbon isotopic fractionation factors ($\varepsilon_c$) for the mixed C1–C4 substrate incubations (Table 1) differ somewhat compared with $\varepsilon_c$ determined from incubations with only one hydrocarbon; the difference is 7.4‰ for C1, 2.6‰ for C2, 0.2‰ for C3, and 0.8‰ for C4. In the case of C1 there is again an apparent discontinuity in the fractionation as substrate levels decrease (at $f \sim 0.25$; equivalent to −0.29 on the $\ln(1-f)$ scale or 0.75% headspace C1), as indicated by the dashed line in Fig. 2E. The apparent fractionation factor associated with the lower C1 level is −11.9 ($r^2 = 0.726$). This fractionation change also corre-

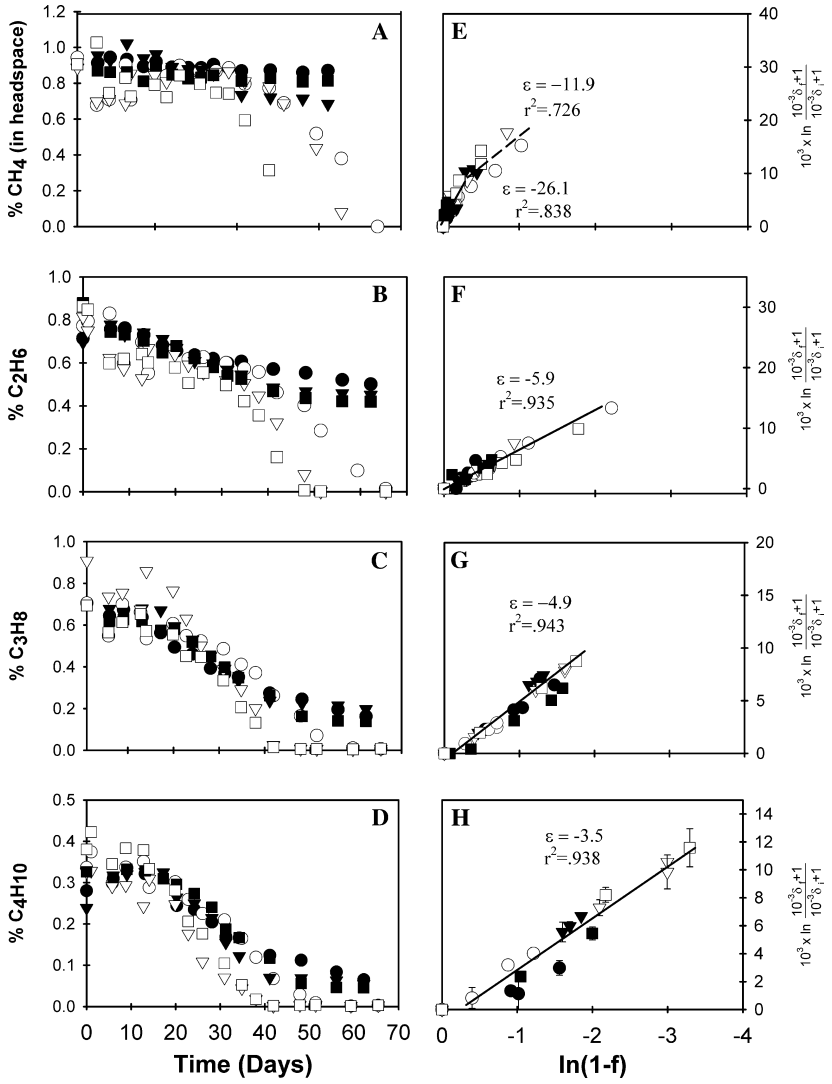


Fig. 2. Incubation of seep sediment with a mixture of hydrocarbon species. (A–D) Display time course changes in headspace C1, C2, C3 and C4, respectively, and plots E–H display the change in $\delta^{13}C$ of the corresponding gas as a function ($\ln(1-f)$) of the fraction consumed. Sets of triplicate samples from each of the two seep environments are displayed on each panel. Solid symbols indicate samples from Shane Seep and open symbols indicate samples from Brian Seep. The $\delta^{13}C$ of each hydrocarbon was analyzed only at select intervals. The dashed line in (E) corresponds to an apparent change in fractionation. Error bars are smaller than the corresponding symbol, except as noted.

sponds to exhaustion of the C2+ hydrocarbons and is noted only in samples from Brian Seep – $O_2$ depletion prevented C1 from being consumed to low levels in samples from Shane Seep. In contrast to the incubations involving only one hydrocarbon in the headspace, there is no apparent discontinuity in fractionation associated with C3.

### 3.3. Fractionation of hydrogen isotopes

A trend toward deuterium enrichment in the residual hydrocarbon gas was observed in experimental treatments concurrent with hydrocarbon consumption. Due to analytical limitations only results from select C1–C3 treatments are available (Table 2).

The range of observed δD values is −86.2‰ to +317.5‰ for C1, −347.0‰ to −252.9‰ for C2, and −143.7‰ to −120.5‰ for C3. As with the carbon isotopic enrichments, fractionation factors were determined in a manner similar to a Rayleigh distillation. $\varepsilon_H$ values averaged from both Shane Seep and Brian Seep incubations were −238.2‰ ± 115.6 for C1, −61.9‰ ± 8.3 for C2, and −15.1‰ ± 1.9 for C3. Of the samples analyzed, one C3 measurement was discarded as it was anomalously depleted relative to all other samples in the time series. Fractionation factors for C1 differed between sediment from the two locations, giving rise to a large standard error in the reported average. The $\varepsilon_H$ value for incubations of sediment taken from Shane Seep was −156.4‰, while that from Brian Seep was −319.9‰. The δD values of residual hydrocarbons were found to correlate with the $\delta^{13}C$ of the corresponding sample. Fig. 3 presents a plot of $\delta^{13}C$ versus δD (C–D plot), inclusive of all samples for which both analyses were performed. The slopes of the linear regressions are indicative of the simultaneous

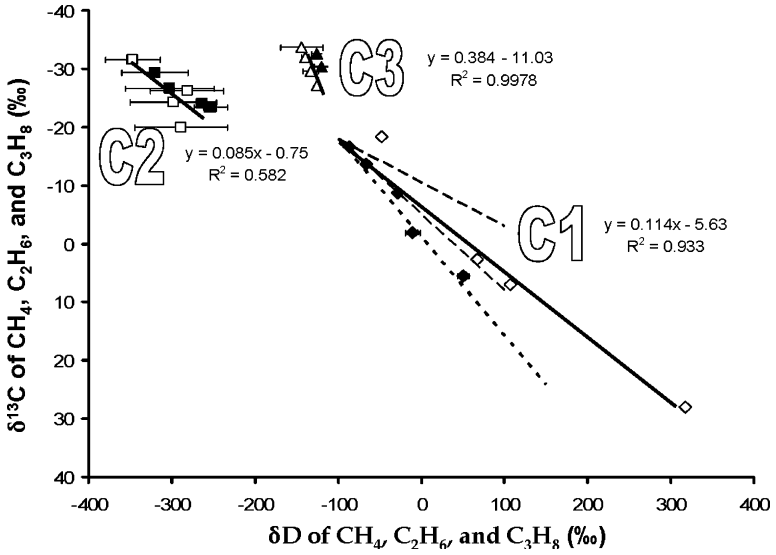


Fig. 3. Simultaneous enrichment in δD and $\delta^{13}C$ (C/D plot) for C1–C3 during microbial consumption. Solid symbols denote incubations originating from Shane Seep, while open symbols denote incubations originating from Brian Seep. Temporal trajectories trend towards the lower right corner. Short dashed lines indicate the approximate trajectory predicted for methanotrophy by Whiticar (1999), and long dashed lines indicate the trajectory predicted by Coleman et al. (1981). For C1 and C2 the solid black lines and corresponding equations incorporate data from both seep locations. For C3 separate regressions were performed for the two seep locations due to differing initial δD values, and the average of the two regressions is provided. Error bars are smaller than the corresponding symbol, except as noted.

Table 1
Carbon isotope fractionation factors ($\varepsilon_c$) for the microbial oxidation of C1–C4 in units of ‰[a]

| | Individual gas incubations[b] | | | | Gas mixture incubations[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | Shane seep | | Brian seep | | Shane seep | | Brian seep | |
| | $\varepsilon$ | $r^2$ | $\varepsilon$ | $r^2$ | $\varepsilon$ | $r^2$ | $\varepsilon$ | $r^2$ |
| $CH_4$ | −25.1 | 0.948 | −25.8 | 0.931 | −33.3 | 0.622 | −19.7 | 0.721 |
| | −26.5 | 0.816 | −25.4 | 0.989 | −25.8 | 0.935 | −32.0 | 0.702 |
| | −28.3 | 0.869 | −23.6 | 0.924 | −50.5 | 0.902 | −31.7 | 0.858 |
| Average | −26.6 ± 1.6 | | −24.9 ± 1.2 | | −36.5 ± 12.7 | | −27.8 ± 7.0 | |
| | $\varepsilon_{pure}$ average: −25.8 ± 1.6 | | | | $\varepsilon_{mix}$ average: −32.2 ± 10.3 | | | |
| $C_2H_6$ | −9.8 | 0.983 | −8.9 | 0.996 | −8.1 | 0.800 | −6.2 | 0.989 |
| | −11.2 | 0.992 | −8.2 | 0.998 | −6.6 | 0.919 | −7.9 | 0.990 |
| | −9.3 | 0.992 | −8.4 | 0.999 | −5.9 | 0.841 | −5.6 | 0.990 |
| Average | −10.1 ± 1.0 | | −8.5 ± 0.4 | | −6.9 ± 1.1 | | −6.6 ± 1.2 | |
| | $\varepsilon_{pure}$ avg. = −9.3 ± 1.1 | | | | $\varepsilon_{mix}$ avg. = −6.7 ± 1.0 | | | |
| $C_3H_8$ | −4.0 | 0.993 | −5.4 | 0.976 | −4.8 | 0.948 | −3.9 | 0.983 |
| | −5.9 | 0.999 | −5.6 | 0.971 | −5.8 | 0.994 | −5.1 | 0.997 |
| | −3.0 | 1 | −5.2 | 0.957 | −4.2 | 0.986 | −5.0 | 0.998 |
| Average | −4.3 ± 1.5 | | −5.4 ± 0.2 | | −4.9 ± 0.8 | | −4.7 ± 0.7 | |
| | $\varepsilon_{pure}$ avg. = −4.9 ± 1.1 | | | | $\varepsilon_{mix}$ avg. = −4.8 ± 0.7 | | | |
| $C_4H_{10}$ | −1.3 | 0.844 | −3.4 | 0.988 | −2.2 | 0.634 | −3.5 | 0.974 |
| | −1.6 | 0.880 | | | −3.5 | 0.999 | −3.4 | 0.996 |
| | −3.3 | 0.965 | | | −2.8 | 0.992 | −3.6 | 0.997 |
| Average | −2.4 ± 1.0 | | −3.4 | | −2.8 ± 0.7 | | −3.5 ± 0.1 | |
| | $\varepsilon_{pure}$ avg. = −2.4 ± 1.1 | | | | $\varepsilon_{mix}$ avg. = −3.2 ± 0.6 | | | |

[a] $\varepsilon_c$ for C1–C4 are reported in the abstract as averages of the $\varepsilon_{pure}$ and $\varepsilon_{mix}$ values (excluding Shane Seep for C1) and are: −26.5‰ ± 3.9 for C1, −8.0‰ ± 1.7 for C2, −4.8‰ ± 0.9 for C3, and −2.9‰ ± 0.9 for C4.
[b] Refers to fractionation factors from incubations involving only one hydrocarbon gas ($\varepsilon_{pure}$).
[c] Refers to fractionation factors from incubations containing mixtures of C1–C4 ($\varepsilon_{mix}$).

enrichment in $\delta^{13}C$ and δD associated with aerobic microbial C1–C3 oxidation. These C/D enrichment factors (in units of ‰$\delta^{13}C$/‰δD) are calculated to be 0.114 for C1, 0.084 for C2, and 0.384 for C3. Whiticar (1999) discussed theoretical shifts in $\delta^{13}C_{CH_4}$ and $\delta D_{CH_4}$ as a result of C1 oxidation and projected a C and H isotope ratio trajectory of approximately 0.165 (dotted line on Fig. 3). Coleman et al. (1981) measured trajectories for two cultures

Table 2
Hydrogen isotope fractionation factors ($\varepsilon_h$) for C1–C3 in units of ‰

| | Shane seep | | Brian seep | | |
|---|---|---|---|---|---|
| | $\varepsilon_h$ | $r^2$ | $\varepsilon_h$ | $r^2$ | Average $\varepsilon_h$ |
| $CH_4$ | −156.4 | 0.936 | −319.9 | 0.991 | −238.1 ± 115.6 |
| $C_2H_6$ | −67.8 | 0.796 | −56.0 | 0.686 | −61.9 ± 8.3 |
| $C_3H_8$ | −13.8 | 1.0 | −16.5 | 0.928 | −15.1 ± 1.9 |

at different temperatures which ranged from 0.13 to .07 (dashed lines on Fig. 3).

## 4. Discussion

### 4.1. Microbial consumption of C1–C4 hydrocarbons

Results from the experiments presented here clearly demonstrate the aerobic consumption of C1–C4 hydrocarbons by natural microbial communities inhabiting shallow marine gas seeps. While the microbial consumption of C1, or methanotrophy, has been the subject of numerous investigations, the microbial oxidation of C2 (ethanotrophy), C3 (propanotrophy) and C4 (butanotrophy) has received little attention—particularly with regard to their occurrence in nature. The study sites in this work were chosen because the ebullition of thermogenic hydrocarbon gas from the sediments into oxic waters led us to hypothesize C1–C4 oxidation was occurring. The consumption of C1–C4 compounds in fresh sediment slurries supports this hypothesis.

Methanotrophs are known to be active around marine gas seeps, and several previous reports detail aspects of their distribution and activity (Bidle et al., 1999; Lanoil et al., 2001; Valentine et al., 2001; Joye et al., 2004). Many methanotrophs are able to cometabolize higher alkanes, but require the presence of C1 for growth (Berthe-Corti and Fetzner, 2002). Results presented here (Figs. 1 and 2) clearly demonstrate the sustained consumption of C2–C4 independent of C1. Results from mixed-hydrocarbon treatments further indicate that C3 and C4 are largely consumed prior to C1 and C2 being consumed, with some indication that C2 is also consumed prior to C1. This preferential oxidation of C2–C4 hydrocarbons was noted in treatments from both seep environments. The reason for this apparent preference is not clear, and may be related to any of several factors including the substrate preferences for different members of the microbial community, the expression of different enzyme systems within an organism, enzymatic affinity for different gases, competition for substrates and nutrients, or to the inherent metabolic capacity of the sediment samples. On purely thermodynamic grounds, the oxidation of C3 and C4 is favored over C1 and C2. While alkanes are among the least chemically reactive organic compounds, C3 and C4 contain C–H bonds at the secondary position (at the non-terminal carbon site) which are more reactive than C–H bonds at the primary position (e.g., C1 and C2). In non-biological reactions, such as gas phase reactions of alkanes with OH, the C3 reaction is approximately 175 times faster than the C1 reaction at 25 °C (Bryukov et al., 2004).

Details regarding the microbes responsible for oxidation of C2–C4 in seep environments are lacking, though microbes capable of growth on C2 (Dworkin and Foster, 1958), C3 (Perry, 1980; Stephens and Dalton, 1986), and C4 (Takahashi, 1980; Hou et al., 1983; Hamamura et al., 1999) have been isolated from other environments. The identity and activity of the organisms responsible for C2–C4 oxidation in seep environments is a subject of ongoing research in our lab, and several strains have been isolated from the incubations described here (Redmond and Valentine, 2005).

### 4.2. Fractionation of stable isotopes

#### 4.2.1. Methane

Numerous studies have reported carbon isotope fractionation factors for C1. Those reported here are slightly larger than many previously reported values (compiled in Reeburgh, 2003; see also Grant and Whiticar, 2002 and Templeton et al., 2006). These results agree best with those obtained in incubations performed ~10 °C warmer (Barker and Fritz, 1981; Coleman et al., 1981). No fractionation factors have been previously reported for sediment gathered from hydrocarbon cold seep environments. The high level of fractionation observed in this study is consistent with $\delta^{13}C$–$CH_4$ enrichments of as much as 60‰ found in sediment surrounding Shane Seep (Kinnaman and Valentine, 2004).

Carbon isotope fractionation was inconsistent for C1 during early stages of the mixed gas incubations. Table 1 displays notably high values of $\varepsilon_c$ for two of the incubations originating from Shane Seep. Methane consumption ceased prematurely in these incubations due to limitation by $O_2$, resulting in only a small change in $f$ over the course of the incubation. Large changes in $f$ are needed to accurately determine $\varepsilon_c$, and may explain the high values reported in Table 1. For this case, considering the aggregate data (Fig. 2E) seems a more accurate approach. Therefore $\varepsilon_c$ for C1 calculated from the mixed C1–C4 incubations originating from Shane Seep were not assimilated into an overall average as was done for C2–C4 incubations. Fractionation factors determined from other individual treatments (Table 1) were otherwise consistent with the fractionation determined from the aggregate data.

The deuterium fractionation factors for C1 reported in Table 2 fall within the range of previously reported fractionation factors. Coleman et al. (1981) report $\varepsilon$ values of $-103 \pm 6$ and $-297 \pm 2$ for incubations at 11.5 and 26 °C, respectively, for an aerobic culture taken from a freshwater well. Snover and Quay (2000) report hydrogen isotope effects of $-99 \pm 30$ for oxidation of atmospheric levels of C1 in grasslands and $-66 \pm 7$ for forest soils.

#### 4.2.2. Ethane, propane and butane

No carbon or hydrogen isotope fractionation factors have been reported for microbial consumption of C2–C4 alkanes

to our knowledge. Previous investigations of hydrocarbon biodegradation in natural environments have focused on tracing organic contaminant sources and fates in geochemical systems and have resulted in reports of fractionation factors for other multi-carbon substrates. Examples of these studies include the anaerobic biodegradation of toluene by microbes from soil ($\varepsilon_c = -1.5 \pm 0.2$; Meckenstock et al., 1999), the aerobic biodegradation of benzene by *Acinetobacter* and *Burkholderia* sp. ($\varepsilon_c = -1.46 \pm 0.06$ and $\varepsilon_h = -12.8 \pm 0.7$ for *Acinetobacter*; $\varepsilon_c = -3.53 \pm 0.26$ and $\varepsilon_h = -11.2 \pm 1.8$ for *Burkholderia*: Hunkeler et al., 2001), and the aerobic biodegradation of vinyl chloride and trichloroethylene ($\varepsilon_c = -5.7 \pm 1.1$ and $-1.1 \pm 0.3$, respectively; Chu et al., 2004). Stable carbon and hydrogen isotopic fractionation of *n*-alkanes ($C_{15}$–$C_{18}$) has only been observed during heavy biodegradation (Sun et al., 2005). The fractionation factors for C2–C4 from this work are generally greater than for the larger molecules as outlined above, though the intrinsic carbon isotope effect (see Morasch et al., 2004) appears similar for C3 ($-14.4 \pm 2.7$), C4 ($-11.6 \pm 3.6$), toluene ($-10.5 \pm 1.4$), benzene ($-8.76 \pm 0.36$—for *Burkholderia*) and trichloroethylene ($-11.4 \pm 2.2$).

#### 4.2.3. Variable fractionation

Variability in the extent of isotope fractionation was observed both between treatments originating from different locations and within individual treatments. Three of the experimental treatments (one involving only C1, one involving only C3, and one involving C1 in a C1–C4 mixture) exhibit apparent discontinuities in isotope fractionation near the end of the incubation (Figs. 1, 2E). In the single hydrocarbon incubations (Fig. 1) these features were interpreted from samples which were below −2.0 on the ln $(1 - f)$ scale, equivalent to 0.4% headspace for C1 (5 samples) or to 0.03% for C3 (3 samples), and also correspond to slower rates of oxidation and decreased oxygen levels. For the mixed gas incubation this feature was interpreted from 9 samples which were below 0.7% C1 headspace. These fractionation factors are illustrated with a dashed line in Figs. 1 and 2, and each is smaller than the corresponding fractionation determined using data collected earlier in the experiment. This discontinuity was not consistent across all experimental treatments. For example, no variation in isotope fractionation was observed for C3 in the mixed gas incubations. Incubations involving C2 and C4 did not display a discontinuity.

Hydrogen isotope fractionation was found to differ for C1 between sediments from the two seep environments. The sediment originating from Brian Seep displayed an $\varepsilon_h$ of −319.9 while that from Shane Seep displayed an $\varepsilon_h$ of −156.4. The underlying cause for this difference is not apparent from the experimental results. This magnitude of variability in hydrogen isotope fractionation has been observed previously, associated with both the consumption (Coleman et al., 1981) and production (Valentine et al., 2004) of C1.

Variable isotope fractionation has been observed in other systems, and a variety of potential causes have been considered (Rees, 1973; Gelwicks et al., 1994; Hall et al., 1999; Hayes, 2001; Valentine et al., 2004; Templeton et al., 2006). Different pure cultures can show great variations in fractionation for similar biological processes. Morasch et al. (2002) found varying levels of carbon isotope fractionation during aerobic toluene degradation for bacterial strains with different enzyme mechanisms. Hall et al. (1999) concluded that the observed variation in fractionation during aerobic degradation of phenol and benzoate was due to differences in bacterial metabolic pathways and growth rates. Conflicting reports of the amount of fractionation occurring for polycyclic aromatic hydrocarbons (PAH's), summarized by Abrajano et al. (2004) imply that enzymes catalyzing similar reactions in different organisms display dissimilar fractionation factors. This is comparable to photosynthesis, where plants with $C_3$, $C_4$ and crassulacean acid metabolism (CAM) pathways exhibit different levels of $^{13}C$ fractionation during $CO_2$ fixation. For the aerobic oxidation of C1–C4, the first step in the biochemical pathway is presumably a uni-directional reaction (e.g., an irreversible oxidation; (Stephens and Dalton, 1986; Hanson and Hanson, 1996; Hamamura et al., 1999; Johnson and Hyman, 2006), and thus the isotopic fractionation must be determined at or prior to this step. The discontinuity in $\varepsilon_c$ observed in this study is consistent with a transport-limited situation. The extreme case of transport-limitation occurs when a cell goes from having bi-directional substrate flux (e.g., substrate exchanges rapidly in and out of the cell relative to biochemical activation) to having uni-directional substrate flux (e.g., all substrate entering the cell is consumed). In the case of bi-directional substrate flux the concentration and isotopic composition of the substrate is influenced by the fractionation associated with the transport into and out of the cell and the isotopic fractionation associated with the biological process, while in uni-directional flow the biological isotopic fractionation is zero and the only fractionation expressed is during transport into the cell. In actuality these two extreme scenarios represent end-members of a continuum, with substrate concentration, transport rates and cell-specific catabolism as the primary controls. While this biochemical explanation is consistent with the experimental observations, other factors may also be important including oxygen levels and shifts in the structure of the microbial community. For example, differences in C3 fractionation between the C3-only incubations and mixed gas incubations may be due to differences in the microbial populations, or to the impact of cometabolism. Differences in the rate of hydrocarbon oxidation (as interpreted in the time course changes in C1–C4 hydrocarbons in Figs. 1 and 2) between replicate incubations do not seem to cause variations in fractionation in these results.

#### 4.2.4. Application of fractionation factors to natural systems

Distributions of $\delta^{13}C$ and $\delta D$ in hydrocarbon gases have previously been applied to approximate the extent of hydrocarbon oxidation. This approach is well established for C1 (Schoell, 1988; Whiticar, 1999), but less so for

C2–C4 (James, 1983; Boreham et al., 2001; Plummer et al., 2005). The fractionation factors presented in this work are specific to oxic marine sediments at 15 °C, and care must be taken in applying these values to other environmental systems. Specifically, temperature, hydrocarbon distributions and dominant terminal electron-accepting processes may each impact the magnitude and expression of isotopic fractionation in a given environment. The reported variability in carbon isotope fractionation associated with C1 oxidation (Reeburgh, 2003) in different environments serves as such an example. The fact that fractionation factors presented here for C2–C4 were similar between two distinct seep environments is promising, but does not guarantee uniformity in anoxic, terrestrial or subsurface environments. Despite these shortcomings we encourage the application of these fractionation factors to estimate the extent of C2–C4 oxidation in different systems, with an understanding of the associated caveats, and the realization that future investigations may refine the relevant fractionation factors for different settings.

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