Subterranean Karst Environments as a Global Sink for Atmospheric Methane

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Abstract: The air in subterranean karst cavities is often depleted in methane (CH4) relative to the atmosphere. Karst is considered a potential sink for the atmospheric greenhouse gas CH4 because its subsurface drainage networks and solution-enlarged fractures facilitate atmospheric exchange. Karst landscapes cover about 14 % of earth’s continental surface, but observations of CH4 concentrations in cave air are limited to localized studies in Gibraltar, Spain, Indiana (USA), Vietnam, Australia, and by incomplete isotopic data. To test if karst is systematically acting as a global CH4 sink, we measured the CH4 concentrations, δ13CCH4, and δ2HCH4 values of cave air from 33 caves in the USA and three caves in New Zealand. We also measured CO2 concentrations, δ13CCO2, and radon (Rn) concentrations to support CH4 data interpretation by assessing cave air residence times and mixing processes. Among these caves, 35 exhibited subatmospheric CH4 concentrations in at least one location compared to their local atmospheric backgrounds. CH4 concentrations and δ13CCH4 and δ2HCH4 values suggest that microbial methanotrophy within caves is the primary CH4 consumption mechanism as the atmosphere exchanges with subsurface air. The pattern of δ13CCH4 and δ2HCH4 values along CH4 concentration gradients in cave air provides evidence for incomplete oxidation by methanotrophy. Only 5 locations from 3 caves showed elevated CH4 concentrations compared to the atmospheric background and could be ascribed to local CH4 sources from sewage and outgassing swamp water. Several associated δ13CCH4 and δ2HCH4 values point to carbonate reduction and acetate fermentation as biochemical pathways of limited methanogenesis in karst environments and suggest that these pathways occur in the environment over large spatial scales. Our data show that karst environments function as a global CH4 sink. Estimates of CH4 flux in karst landscapes are needed in order to include the subterranean CH4 sink in climate models.

Keywords: cave; greenhouse gas; karst; methane; methanogenesis; methanotrophy
1. Introduction

Atmospheric methane (CH₄) is a greenhouse gas and its concentration is increasing in the atmosphere (Dlugokencky et al., 2011; Sussmann et al., 2012; Ciais et al., 2013). The increase in atmospheric CH₄ is due to an imbalance between CH₄ sources and sinks. Anthropogenic and natural sources combine to contribute about 680 Tg of CH₄ to the atmosphere while reactions with hydroxyl (·OH) and chlorine radicals in the troposphere and stratosphere remove about 600 Tg a⁻¹ (Kirschke et al., 2013). Methanotrophic consumption in soils removes 30 Tg a⁻¹ (Kirschke et al., 2013). The present globally averaged CH₄ concentration is 1.87 ppmv which is 2.5 times higher than preindustrial levels (Nisbet et al., 2016). Despite improvements in estimating individual sources and sinks of atmospheric CH₄, the associated errors remain large (Kirschke et al., 2013). Recent studies suggest that caves may act as an additional CH₄ sink (Mattey et al., 2013; Fernandez-Cortes et al., 2015; McDonough et al., 2016; Webster et al., 2016; Lennon et al., 2017).

Caves and associated karst landscapes may be an important overlooked sink for atmospheric CH₄ because they are estimated to cover as much as 10 to 20 % of the continental surface with the more precise estimates suggesting about 13.8 % (Palmer, 1991; Ford and Williams, 2007). Karst landscapes are frequently associated with the chemical dissolution of limestones, but can form in any soluble rock body. The resulting caves, solution-enlarged fractures, and internal drainage networks that function to transport mass from high elevations to low elevations also allow for subsurface-surface atmospheric exchange (Kowalczk and Froelich, 2010; Garcia-Anton et al., 2014). The total volume and surface area of karst conduits able to interact with the atmosphere is unknown, in part due to small fractures and the difficulty of imaging the subsurface with geophysical methods. Karst caves, due to their accessibility, provide opportunities for non-invasive, in-situ analyses and sampling.

Cave and karst landscapes form in two common ways, each of which influences karst’s capacity to act as a CH₄ sink. Epigenic karst forms through the interaction of limestone with carbonic acid derived from the dissolution of atmospheric and soil CO₂ into surface waters, and hypogenic caves form when corrosive water from deep sources migrates into and dissolves limestone bedrock. Epigenic caves are more widespread, and atmospheric to subatmospheric CH₄ concentrations of 1.8 ppmv to < 0.1 ppmv have been observed in these settings (Mattey et al., 2013; Fernandez-Cortes et al., 2015; McDonough et al., 2016; Webster et al., 2016; Lennon et al., 2017). In some hypogenic caves be contrast, elevated CH₄ concentrations from 2 ppmv to 1 % have been observed in association with CH₄-rich springs or seeps related to fluid migration from deep hydrocarbon-bearing sedimentary rocks, i.e. seepage processes that are widespread on Earth (Sarbu et al., 1996; Hutchens et al., 2004; Jones et al., 2012; Webster et al., 2017). The dominance of epigenic karst suggests that karst is likely functioning as a CH₄ sink at the global scale, but more observations are needed.

Different hypotheses have been put forward to explain the low CH₄ concentrations observed in epigenic cave air. The combination of subatmospheric CH₄ concentrations and the stable carbon isotopic ratio of CH₄ in the air of caves in Gibraltar led to the hypothesis that microorganisms were responsible for the removal of CH₄ (Mattey et al., 2013). In turn, low CH₄ concentrations in Spanish caves, in the presumed absence of CH₄-consuming (methanotrophic) bacteria, led to the hypothesis that CH₄ oxidation was induced by ions and ·OH generated by the radioactive decay of radon and daughter nuclides (Fernandez-Cortes et al., 2015). Since these initial observations, datasets from caves in Australia, the USA, and Vietnam have pointed towards methanotrophic CH₄ oxidation (McDonough et al., 2016; Webster et al., 2016; Lennon et al., 2017).

The chemical composition of cave air results from the mixing of the atmosphere and air from the overlying soils and epikarst and these processes should influence the CH₄ concentrations of cave air. Previous studies have shown that CH₄ concentrations have been inversely correlated with CO₂ concentrations in cave air (Mattey et al., 2013; Fernandez-Cortes et al., 2015; McDonough et al., 2016;
Webster et al., 2016). Cave air CO₂ concentrations are positively correlated with radon (Rn) concentrations and Rn is known to track cave air residence time (Cunningham and LaRock, 1991; Batiot-Guilhe et al., 2007; Kowalczyk and Froelich, 2010; Matthey et al., 2010; Gregorič et al., 2011, 2014). Additionally, the stable C isotope composition of CO₂ (δ¹³CCO₂), can track the sources of CO₂ in the environment. For example, δ¹³CCO₂ values of −24 ‰ are associated with soil CO₂, while atmospheric CO₂ has δ¹³CCO₂ values ranging from −8.5 ‰ to −10 ‰ (Amundson et al., 1998; Keeling et al., 2010; Peyraube et al., 2013). Thus CO₂, Rn, and δ¹³CCO₂ in cave air can help determine the influence of cave air mixing processes on CH₄.

The stable C and H isotope compositions of CH₄ (δ¹³CCH₄ and δ²HCH₄) also provide tools for understanding the sources and sinks of CH₄ in caves because different CH₄ sources are associated with characteristic δ¹³CCH₄ and δ²HCH₄ values. For example, CH₄ produced from carbonate reduction has δ¹³CCH₄ and δ²HCH₄ values that range from −112 to −60 ‰ VPDB and from −350 to −100 ‰ VSMOW respectively (Whiticar, 1999), and atmospheric CH₄ has δ¹³CCH₄ and δ²HCH₄ values around −47.5 and −100 ‰ (Miller et al., 2002; Townsend-Small et al., 2012). The δ¹³CCH₄ and δ²HCH₄ values of CH₄ can also be altered through secondary processes such as oxidation and mixing. The oxidation pathways of CH₄ by methanotrophs or -OH have fractionation factors that result in residual CH₄ having increased in δ²HCH₄ values of 8.5 ‰ for every 1 ‰ increase in δ¹³CCH₄ value and increases in δ²HCH₄ values of 72 ‰ for every 1 ‰ increase in δ¹³CCH₄ value, respectively (Feisthauer et al., 2011; Sauereissig et al., 2001). Mixing between two different CH₄ sources creates a linear trend between the two members. Thus measuring the δ¹³CCH₄ and δ²HCH₄ of cave air should allow for the determination of cave air CH₄ sources.

The objective of the present work is to extend the karst CH₄ dataset and test the hypothesis that karst systems act as a CH₄ sink on a global scale. To this aim, we studied CH₄ concentration, δ¹³CCH₄, and δ²HCH₄ in cave air from 33 epigenic caves in the USA and three epigenic caves in New Zealand. CO₂, δ¹³CCO₂, and Rn were also measured to support CH₄ data interpretation via assessing cave air residence times and mixing processes. Data analysis is focused on determining CH₄ concentrations, origin, mixing processes and isotopic fractionations.

2. Methods

2.1. Sampling and analyses

Air samples from 16 limestone or dolostone caves in the Appalachian fold and thrust belt, 17 limestone caves in gently warped intracratonic basins of the USA, and 3 caves from the North Island of New Zealand were collected over a timespan of roughly four years (Fig. 1; Table 1). Cave air was analyzed using in-situ methods and was further sampled for laboratory analysis. In-situ CH₄, CO₂, and Rn abundance analyses were carried out using a suite of instruments (Table 2). Discrete samples of cave air were collected in pre-evacuated 50-mL serum vials, in 1 to 3-L Tedlar® bags, or in 4-L glass bottles. CH₄ and CO₂ concentrations of discrete samples were measured via gas chromatography.

We assessed cave air mixing processes through the following techniques. A qualitative estimate on cave air residence time was obtained by comparing CH₄ to CO₂ concentrations at individual locations in each cave. Additionally, we measured the Rn concentrations of caves 32 through 36 to assess the relationship between cave air residence time, CH₄ concentrations, and CO₂ concentrations. δ¹³CCO₂ data were used to assess the sources of CO₂ and thus of air entering the caves. We also assessed the distance from each sampling location to cave entrances as another tool to understand the sources and sinks of CH₄ in caves.

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CH4 and CO2 concentrations from discrete air samples were measured at Indiana University using a Varian 450 gas chromatograph (GC) (Varian – Agilent Technologies, Palo Alto, California). The GC was fitted with a flame ionization detector (FID) for CH4 and a thermal conductivity detector (TCD) for CO2. Standard gas mixtures from Air Liquide America Specialty Gasses LLC (Plumsteadville, Pennsylvania) were used for 3-point calibration curves to convert signals measured on the GC to concentrations. CH4 concentrations are reported with the uncertainty associated with the standard curve unless the calculated uncertainty was ≤ 0.1 ppmv. Samples with calculated uncertainties ≤ 0.1 ppmv were assigned uncertainties of 0.1 ppmv based on replicate measurements. The uncertainty associated with standard curves for CO2 concentrations varied from < ± 1 to 5 %. CO2 concentrations were assigned uncertainties based on their associated standard curve.

The stable carbon isotope ratios of CH4 and CO2 and hydrogen stable isotope ratios of CH4 were measured on a ThermoFinnigan Delta Plus XP mass spectrometer in the Stable Isotope Research Facility at Indiana University. Carbon stable isotope ratios are expressed as conventional δ13CCH4 and δ13CCO2 values in ‰ along the scale anchored to Vienna Pee Dee Belemnite (VPDB). Hydrogen stable isotope ratios are expressed as δ2HCH4 values in ‰ along the scale anchored to Vienna Standard Mean Ocean Water (VSMOW). CH4 samples were measured in continuous-flow mode using CH4 preconcentration, cryofocusing (Miller et al., 2002), and a gas chromatography-oxidation/pyrolysis-isotope ratio mass spectrometer (GC-ox/pyr-IRMS) interface. Varying sample extraction times were used to isolate roughly 0.45 and 0.90 μmol of CH4 prior to the introduction of the sample to the GC-ox/pyr-IRMS for analysis of δ13CCH4 and δ2HCH4 values, respectively. In-house CH4 standards methane #3, methane #6, and methane ALM with δ13CCH4 and δ2HCH4 values of [+19.86 ± 0.05; +2.2 ± 1.2] ‰, [−39.40 ± 0.02; −153 ± 2] ‰, and [−58.6 ± 0.03; −272.2 ± 3.4] ‰ were used for 2-point normalizations. Errors associated with δ13CCH4 and δ2HCH4 values were calculated using a standard curve that accounted for the peak size of the measurement. Analytical repeatability of internal standards ranged from 0.14 to 0.6 ‰ for δ13CCH4 and from to 7 to 18 ‰ for δ2HCH4.

δ13CCO2 values were measured in continuous-flow mode using a GasBench II inlet (Tu et al., 2001). Measured 13C/12C ratios of CO2 from cave air were converted to the VPDB scale using a single isotopically characterized in-house standard that has a value of 12.0 ± 0.2 ‰.

2.2 Data elaboration and quality control

In-situ measurements were preferentially used when statistically analyzing gas concentration data. When in-situ measurements were not available, concentrations measured on the GC were used in the statistical analyses. Samples and data were screened for quality control by comparing the samples with in-situ measurements and visual estimation of the volume of sample bags. If a sample bag had been shown to exhibit a leak for one analyte, data from that sample were discarded. CH4 and CO2 concentrations measured by both GC-FID and FTIR showed strong agreements (GC(CO2) = 0.92 ± 0.04 * FTIR(CO2) + 100 ± 300, r2 = 0.99, p = 5*10−19; GC(CH4) = 0.7 ± 0.2 * FTIR(CH4) + 0.2 ± 0.2, r2 = 0.62), and the stable isotopic composition of the samples was not related to their storage time (δ13CCH4 = 0.2 ± 0.3*day −47 ± 2, r2 = 0.03, p = 0.32; δ2HCH4 = 0.03 ± 0.14*day −96 ± 11, r2 = 0.005, p = 0.72; δ13CCO2 = −0.05 ± 0.16*day −19.9 ± 1.8, r2 = 0.006, p = 0.57). In locations where more than one sample was taken with in-situ methods, the values of the samples were averaged. In total, 199 CH4 concentrations, 192 CO2 concentrations, 32 δ13CCH4 values, 26 δ2HCH4 values, and 60 δ13CCO2 values are reported in this study (Supplemental Tables 1, 2). All samples are reported with 95 % confidence intervals.
Two different modeling techniques were used to assess trace gas sources and sinks in the studied caves. Keeling plots were used to assess the possibility of a two end member mixing system affecting $\delta^{13}$CCO$_2$. The stable isotopic composition of CO$_2$ entering the caves ($\delta^{13}$C$_{\text{atm}}$) was assessed through equation 1

$$\delta^{13}C = (\delta^{13}C_{\text{m}} - F_{\text{atm}} \cdot \delta^{13}C_{\text{atm}}) (1 - F_{\text{atm}})^{-1}$$  

where $\delta^{13}C_{\text{m}}$ is the $\delta^{13}$CCO$_2$ of the sample, $\delta^{13}C_{\text{atm}}$ is the $\delta^{13}$CCO$_2$ of the atmosphere, and $F_{\text{atm}}$ is the fraction of atmospheric CO$_2$ in the CO$_2$ concentration of the sample (Peyraube et al., 2016). We used values of $-10$ ‰ for $\delta^{13}C_{\text{atm}}$ and 400 ppmv for the concentration of atmospheric CO$_2$.

Rayleigh distillation models were used as the theoretical basis to examine changes in the stable isotopic composition of CH$_4$ in cave air caused by methanotrophy or $\cdot$OH oxidation. The $\delta$-value of an isotope system in a chemical compound of interest (e.g., CH$_4$) in cave air can be modeled as

$$\delta_i = (\delta_i + 1000) f^{-2 \alpha + 1} - 1000$$  

where $\delta_i$ is the instantaneous $\delta$-value of a particular isotope system in cave air after partial consumption, $\delta_i$ is the initial $\delta$-value of the isotope system in cave air, $f$ is the fraction of the compound remaining, and $\alpha$ is the kinetic isotope fractionation factor (Mattey et al., 2013). $\alpha$ values of 1.018 and 1.1353 were used to model changes in $\delta^{13}$CCH$_4$ and $\delta^{2}$HCH$_4$ caused by methanotrophy (Coleman et al., 1981; Feisthauer et al., 2011). $\alpha$ values for changes in $\delta^{13}$CCH$_4$ caused by methanotrophy have been observed to range from 1.003 to 1.039 (Templeton et al., 2006; Feisthauer et al., 2011); 1.018 was selected based on observations of methanotrophy in soils and its similarity to the $\alpha$ value of 1.012 observed in St. Michael’s Cave in Gibraltar (Feisthauer et al., 2011; Mattey et al., 2013). $\alpha$ values of 1.0039 and 1.294 were used to model changes in $\delta^{13}$CCH$_4$ and $\delta^{2}$HCH$_4$ values caused by $\cdot$OH oxidation (Saueressig et al., 2001). The initial stable isotopic composition of atmospheric CH$_4$ was modeled with a $\delta^{13}$CCH$_4 = -47.5$ ‰ (VPDB), and $\delta^{2}$HCH$_4 = -100$ ‰ (VSMOW) based on the work of Townsend-Small et al. (2012).

### 3. Results

Each of the 36 caves showed atmospheric to subatmospheric CH$_4$ concentrations in at least one location. Only five locations from three different caves showed elevated CH$_4$ concentrations relative to the surface atmosphere (Fig. 2, Supplemental Table 1). The CH$_4$ concentration in the surface atmosphere at study sites ranged from 1.8 ± 0.3 to 2.8 ± 0.7 ppmv. CH$_4$ concentrations in cave air ranged from ≤0.1 ± 0.1 ppmv to 5 ± 1 ppmv, and were generally observed to decrease with the distance from cave entrances (Fig. 3, Supplemental Table 1). Two thirds of the caves where three or more air measurements and distance data were recorded showed decreases in CH$_4$ concentration from cave entrances to interiors. For example, caves 7, 8, and 9 from Kentucky all showed progressive decreases in CH$_4$ concentration from about 2 ppmv at the entrance of the cave, down to zero or near zero ppmv in the more inner rooms (from 2 to 0 in caves 8 and 9 and from 1.9 to 0.3 ppmv in cave 7). Additionally, CH$_4$ concentrations were negatively correlated with CO$_2$ concentrations in cave air following an inverse power law relationship ($[\text{CH}_4] = 17.5[\text{CO}_2]^{-0.41}$, $r^2 = 0.26$) (Fig. 2). In the caves where Rn concentrations were measured, the average CO$_2$ concentration of cave air was correlated with the average Rn concentration of cave air ($[\text{CO}_2] = (1.42 \pm 0.09)[\text{Rn}] + 400 \pm 120$, $n = 4$, $r^2 = 0.99$, $p = 0.009$).

Values of $\delta^{13}$CCO$_2$ in cave air ranged from $-10.7 \pm 0.4$ to $-23.81 \pm 0.10$ ‰. Analysis of $\delta^{13}$CCO$_2$ values from samples with CO$_2$ concentrations above 600 ppmv showed that $\delta^{13}$C$_{\text{m}}$ ranging from $-28$ ‰ to $-20$ ‰ contributed to the composition of CO$_2$ in cave air. Pooled analysis of the CO$_2$ dataset shows that the average apparent source $\delta^{13}$CCO$_2$ value is $-23.3 \pm 0.5$ ‰ ($\delta^{13}$CCO$_2 = 4600 \cdot [\text{CO}_2]^{-1} - 23.3 \pm 0.5$ ‰, $r^2 = 0.83$) (Fig. 4).
Values of $\delta^{13}$C$_{\text{CH}_4}$ and $\delta^{2}$H$_{\text{CH}_4}$ in cave air ranged from $-57.2 \pm 0.6$ to $-27.1 \pm 0.2$ ‰ and $-196 \pm 10$ to $+2 \pm 18$ ‰, respectively. Keeling plots of $\delta^{13}$C$_{\text{CH}_4}$ did not suggest that a two end member model was an adequate fit for the system ($\delta^{13}$C$_{\text{CH}_4}$ vs. [CH$_4$]): $r^2 = 0.05$, $\delta^{2}$H$_{\text{CH}_4}$ vs. [CH$_4$]: $r^2 = 0.12$). Some cave air samples plotted near the theoretical relationship between $\delta^{13}$C$_{\text{CH}_4}$ values and CH$_4$ concentrations caused by methanotrophy (Fig. 5). However, many points fell below and to the left of the line representing the theoretical incomplete oxidation of atmospheric CH$_4$ ($r^2$). Some points, like those from caves 25 and 26, plotted near the signature of atmospheric CH$_4$ (Fig. 7). Other points plotted below or above this trend and indicated that at least two additional sources of CH$_4$ had to be entering the caves.

4. Discussion

4.1. Subsurface-Surface Atmospheric Exchange

The concentrations and stable isotopic compositions of CH$_4$, CO$_2$, Rn in cave air overlapped and diverged from those of the surface atmosphere. This suggests that atmospheric and cave processes influenced the composition of cave air. The atmospheric CH$_4$ concentration at many study locations was above the globally averaged atmospheric background concentrations (1.87 ppmv CH$_4$; Ciais et al., 2013) probably because they were near roads or pastures with local CH$_4$ sources (Gioli et al., 2012; Harper et al., 2014). Rn concentrations were positively correlated with CO$_2$ concentrations in cave air which agrees with other observations that have shown that CO$_2$ concentrations in cave air track cave air residence time. The majority of cave air samples were depleted in CH$_4$ and enriched in CO$_2$ relative to the atmosphere, pointing to processes like in-situ CH$_4$ oxidation and diffusion of air from the epikarst to decrease CH$_4$ and increase CO$_2$ concentrations (Fig. 2). Additionally, cave air CH$_4$ concentrations generally decreased as the distance from an entrance increased (Fig. 3), and departures from this trend can be explained by fast airflow (caves 13, 25), cave air flowing out of the entrance (cave 26), multiple entrances resulting in multiple flow paths (cave 24), distance scales that were too small to observe a decrease in CH$_4$ concentration (cave 20), or internal CH$_4$ sources (cave 9). The patterns of CH$_4$, CO$_2$, and Rn concentration indicate that CH$_4$ concentrations decrease with cave air residence time.

The concentration and stable isotopic composition of CO$_2$ in cave air show that in addition to an atmospheric end member, CO$_2$ with $\delta^{13}$C$_{\text{CO}_2}$ values ranging from $-28$ to $-20$ ‰ were entering the caves and that the average source $\delta^{13}$C$_{\text{CO}_2}$ value was $-23.3 \pm 0.5$ ‰ (Fig. 4). These observations generally agree with a CO$_2$ source from the soils and an atmospheric end-member. The $\delta^{13}$C$_{\text{CO}_2}$ values of many soils is near $-24$ ‰ (Amundson et al., 1998), and atmospheric $\delta^{13}$C$_{\text{CO}_2}$ values are frequently observed to be near $-10$ ‰ (Peyraube et al., 2013; Fernandez-Cortes et al., 2015). Due to fast airflow in cave 13 in Kentucky, the CO$_2$ concentration of 350 ± 6 ppmv and $\delta^{13}$C$_{\text{CO}_2}$ = $-10.7 \pm 0.4$ ‰ in cave air were similar to the local background atmosphere (i.e. 400 ± 90 ppmv CO$_2$). It is possible that the observed source values that are more negative than $-24$ ‰ may be due to dripwater degassing or fast airflow (Spötl et al., 2005; Garcia-Anton et al., 2014). The $\delta^{13}$C$_{\text{CH}_4}$ values that are more positive than $-24$ ‰ may be caused by differential abundances of C3 and C4 plants above the caves (Breecker et al., 2012). Our CO$_2$ data show that the caves in our study are not atypical compared to other caves in the literature and that cave air residence time increased as the $\delta^{13}$C$_{\text{CO}_2}$ values of cave air decreased.

4.2. Sources and Stable Isotopic Composition of Methane
Subatmospheric CH₄ concentrations in cave air suggest that CH₄ from the outside atmosphere is consumed in caves, and the addition of these observations to the existing datasets shows that karst is behaving as a sink for atmospheric CH₄ (Mattey et al., 2013; Fernández-Cortes et al., 2015; McDonough et al., 2016; Webster et al., 2016; Nguyễn-Thuỷ et al., 2017). Only three caves exhibited CH₄ concentrations above the atmospheric background, namely (i) cave 3, Indiana, (ii) cave 22, Tennessee, and (iii) cave 32, New Zealand. Caves with CH₄ concentrations above the atmospheric background appear to be uncommon, and understanding if there is a systematic change in karst landscapes from smaller order to higher order drainages awaits future work.

In some of the caves where we measured Rn concentrations, cave air flow was relatively fast. The average CO₂ and Rn concentrations from these caves were relatively low (cave 33, [CO₂] = 540 ± 20 ppmv, [Rn] = 20 ± 100 Bq m⁻³; cave 34, [CO₂] = 560 ± 30 ppmv, [Rn] = 200 ± 400 Bq m⁻³). Despite the similarity to atmospheric CO₂ concentrations in these caves, average CH₄ concentrations were still depleted relative to the atmosphere (cave 33, [CH₄] = 1.07 ± 0.06 ppmv; cave 34, [CH₄] = 1.34 ± 0.06 ppmv). These observations show that an in-situ process is removing CH₄ from the cave air because subatmospheric CH₄ concentrations in cave air are still observed in the absence of large increases in CO₂ concentrations as would be expected if CH₄ were only diluted by the arrival of CH₄-free air into caves from soils.

In several caves, values of δ¹³CCH₄ and δ²HCH₄ show that non-atmospheric CH₄ enters caves because they were more negative than those predicted by the oxidation of atmospheric CH₄ via methanotrophy or reaction with ∙OH. Other studies have also shown microbially produced CH₄ entering caves (Mattey et al., 2013; Webster et al., 2016). Our data point to the methanogenic sources of acetate fermentation and carbonate reduction (Fig. 7). Mixing between residual atmospheric CH₄ after partial methanotrophic oxidation and CH₄ from acetate fermentation in the soil-epikarst-cave system will cause a decrease in the δ¹³CCH₄ and δ²H CH₄ values of cave air compared to the atmospheric oxidation curve. Caves 23, 24, and 26 all appear to be influenced by acetoclastic methanogenesis. CH₄ produced from carbonate reduction is inferred to enter the caves based on samples that had δ²HCH₄ values that were roughly equal to, or more positive than atmospheric values (Fig. 7). The stable isotopic compositions of CH₄ from caves 5, 15, 25, and 27 can be explained by partial methanotrophic consumption of CH₄ generated from carbonate reduction with the strongest source signal in cave 5 (Fig. 7). CO₂ reduction is typically observed in lake sediments, but has been observed in oxidizing environments such as biological soil crusts in deserts after rain events (Angel et al., 2011). We hypothesize that karst environments, which are less oxidizing, exhibit similar behavior. Our data show that carbonate reduction and acetate fermentation can occur in similar environments over large spatial scales and are not limited to arctic environments.

Sites of methanogenesis in or near the studied cave systems may include waterlogged soils above caves, cave soils themselves, and the epikarst. It is possible that after rain events anoxic micro niches occur in soil, the epikarst, or caves themselves and that the generated CH₄ is dissolved and later introduced into caves with drip waters. We confirmed that dissolved CH₄ outgasses in drip water of cave 32, which was situated underneath a wetland, by placing our CH₄ detecting probe near the water and measuring increased CH₄ concentrations in its vicinity. We confirmed that in-situ CH₄ production can take place in locally anoxic environments within caves by measuring CH₄ concentrations close to a bat guano deposit in cave 25, Tennessee (site 4h; average CH₄ concentration = 0.3 ± 0.5 ppmv). Time series measurements near the large bat guano deposit showed that CH₄ concentrations oscillated between 0.5 ppmv and 0.1 ppmv over the course of seconds, presumably in response to episodic migration of CH₄ bubbles through the moist guano (similar oscillations in ammonia, NH₃, were also observed). Additionally, we observed circumstantial evidence for local in-situ CH₄ production in cave 3 because measured CH₄ concentrations upstream of a restroom in the cave were low, while measured CH₄ concentrations downstream of the restroom were enhanced. Our data show that caves are capable of expressing elevated CH₄ concentrations.
due to in-situ CH4 production when accumulations of organic matter, such as guano or plant material, foster methanogenesis or when dissolved CH4 from waters outgasses into cave air.

We observed minor amounts of thermogenic CH4 entering in at least one cave. Locally elevated CH4 concentrations in cave 9 (Mammoth Cave, Kentucky) were associated with a known hydrocarbon seep that is also transporting sulfide (Olson, 2013). Elevated CH4 concentrations, thought to derive from thermogenic CH4, have also been observed at sulfidic springs in Cueva de Villa Luz (Webster et al., 2017). Some δ13CCH4 and δ2HCH4 values in cave air, i.e. two of the samples from cave 24, are compatible with thermogenic CH4 isotopic signatures (e.g., Schoell, 1988; Etiope et al., 2009; Fig. 7). It cannot be excluded that small fluxes of CH4 from shales or hydrocarbon deposits underlying the limestone are entering caves through natural fractures, but our present isotope data cannot confirm this source for cave 24. The Antes and Utica shales, which contain hydrocarbon gases, are stratigraphically below cave 24 (Coleman et al., 2014), and geologic faults and joints, which are often aligned with caves, may serve as conduits for the flow of hydrocarbons (Powell, 1969; Koša et al., 2003). A confirmation of hydrocarbons entering from deep sources may be obtained through measurements of ‘radiocarbon-dead’ CO2, Rn, and ethane.

4.3. Methane Oxidation Mechanisms

The combination of δ13CCH4 and δ2HCH4 values allow for inferences to be made about how CH4 oxidizing reactions in karst environments. We distinguish between two scenarios with distinct sets of assumptions. Many isotopic compositions of CH4 in this study cannot be accounted for in the first scenario in which we assume that (i) CH4 enters the caves from the atmosphere, through acetate fermentation, and through carbonate reduction, and that (ii) CH4 is removed from cave air through reactions involving the \( \cdot \text{OH} \). Conversely, in a second scenario, if it is assumed that (i) sources of CH4 in cave air include the atmosphere, acetate fermentation, and carbonate reduction, and that (ii) CH4 is removed from cave air by methanotrophy, all of the points fall within the plausibility envelope of the model, suggesting that methanotrophy is the mechanism responsible for removing CH4 from cave air (Fig. 7). Consideration of an additional source of thermogenic CH4 (natural gas) from deep geologic sources enlarges the plausibility fields of both prior scenarios to encompass all of the data. Our δ13CCH4 and δ2HCH4 data also agree with observations of δ13CCH4 and δ2HCH4 from a cave in Indiana where it appeared that CH4 from both acetate fermentation and carbonate reduction influenced cave air geochemistry (Webster et al., 2016). Additionally our data resemble an arctic system characterized by acetoclastic and hydrogenotrophic CH4 sources and methanotrophy (McCalley et al., 2014). Our isotopic evidence for in-situ microbial CH4 oxidation in caves is corroborated by recent results from in-situ mesocosm experiments in Vietnam where cave rocks with live microorganisms were shown to consume CH4 even in cases where surface soils were very thin to non-existent (Lennon et al., 2017; Nguyễn-Thuý et al., 2017).

5. Conclusions

Subterranean karst air generally shows subatmospheric CH4 concentrations. CH4 and CO2 concentrations were negatively correlated in cave air showing that as the residence time of cave air increases the CH4 concentration of cave air decreases. The stable isotopic composition of CH4 in studied caves suggests that CH4 is being oxidized by microbial methanotrophy. This evidence adds to earlier reports that methanotrophy is the mechanism by which CH4 is removed in cave air (Mattey et al., 2013; McDonough et al., 2016; Webster et al., 2016; Lennon et al., 2017). The observations of sub-atmospheric CH4
concentrations in cave air from this study and other studies shows that karst is behaving as a global sink for CH₄. CH₄ flux data from cave and karst landscapes are needed to estimate the size of the karst sink.

The stable isotopic composition of CH₄ in the studied caves suggests that, in addition to atmospheric CH₄, at least two additional CH₄ sources are present in some caves. We suggest that the sources include CH₄ produced from acetate fermentation and from CO₂ reduction. These data corroborate recent findings of partially oxidized CH₄ entering cave air from acetate fermentation and CO₂ reduction in Indiana (Webster et al., 2016). These observations of CH₄ production by acetate fermentation and carbonate reduction suggest that both processes happen over a wide scale in the environment.

Cave air CH₄ appears to have been isotopically shifted towards more positive δ¹³CCH₄ and δ²HCH₄ values due to methanotrophy in the soil-epikarst-cave system. Even though minor CH₄ production appears to be taking place in karst environments, CH₄ consumption is the dominant process.

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Figure 1. Locations of the U.S. study caves in their regional context (A) and within the contiguous USA (B). The location of the New Zealand caves are presented in C. Karst land cover data were obtained from Weary and Doctor (2014) and Ford and Williams (2007).
Figure 2. CH₄ concentrations versus CO₂ concentrations in studied caves. The majority of samples appear to follow an inverse power law relationship with CH₄ concentrations being inversely related to CO₂ concentrations (with points from caves 3 and 22 removed from the overall trend). Numbers represent individual caves. For clarity some error bars were omitted from the figure. Data associated with error bars are also representative for typical errors of data where no bars are shown.
Figure 3. The spatial variation of CH₄ mole fraction in the air of cave 8, Kentucky. CH₄ concentration dropped sharply from 1.5 to 0.5 ppmv along a narrow path after the first large room, a few tens of meter from the entrance. CH₄ gradually decreased over roughly 100 m down to 0 ppmv in the Cathedral room. Both the Inspectra (Gazomat, France assembled by West Systems, Italy) and the GasFinder2 (Boreal Laser Inc., Spruce Grove, Alberta, Canada) use tunable diode lasers.
Figure 4. A Keeling plot of $\delta^{13}C_{CO_2}$ versus inverse CO$_2$ concentration in cave air samples. The data show that atmospheric CO$_2$ is mixing with apparent isotopic endmembers between $-33$ and $-20.4$ ‰. A regression analysis of the entire data set shows that the average $\delta^{13}C_{CO_2}$ value entering the caves is $-23.3 \pm 0.5$ ‰. Numbers represent individual caves. Error bars are not included for clarity.
Figure 5. Relationship between CH₄ concentration and δ¹³C(CH₄) in cave air. Some samples plot along the expected relationship between CH₄ concentration and δ¹³C(CH₄) caused either by methanotrophy modeled with an α value of 1.018, or by oxidation with -OH modeled with an α value of 1.0039. Other samples plot below and to the left of the theoretical shifts of the oxidation trends. Numbers represent individual caves. If error bars are not visible, they are smaller than the data points.
Figure 6. Relationship between methane concentration and $\delta^{2}H_{CH_{4}}$ in cave air. Some samples plot along the expected relationship between CH$_4$ concentration and $\delta^{2}H_{CH_{4}}$ caused by methanotrophy modeled with an $\alpha$ value of 1.1353, or by oxidation with $\cdot$OH modeled with an $\alpha$ value 1.294. Other samples plot below and to the left of the shift caused by methanotrophy. Numbers represent individual caves. If error bars are not visible, they are smaller than the data points.
Figure 7. Stable isotopic composition of CH₄ in cave air samples plotted in δ²H₃CH₄ versus δ¹³C₃CH₄ space. 

CH₄ generated by carbonate reduction, acetate fermentation, and thermogenesis are plotted within labeled fields (Whiticar, 1999). Dotted lines indicate the expected shift in δ²H₃CH₄ and δ¹³C₃CH₄ caused by partial aerobic methane oxidation adopting a slope of 8.5. Dashed lines indicate the expected shift in δ²H₃CH₄ and δ¹³C₃CH₄ caused by oxidation with -OH adopting a slope 75 (Saueressig et al., 2001; Feisthauer et al., 2011). Mixing in δ²H₃CH₄ vs δ¹³C₃CH₄ space plots as a straight line. Numbers represent individual caves. If the cave system can be characterized by sources from the atmosphere (□, −47.5 ‰, −100 ‰), acetate fermentation (S1, −49 ‰, −325 ‰), and carbonate reduction (S2, −63 ‰, −125 ‰), and methanotrophy the
data should plot between the methanotrophic boundaries between S1 and S2, shaded in medium gray. If the same sources are considered to be entering the cave and are oxidized by the -OH radical they should plot between the -OH oxidized boundary of S1 and the atmosphere, and mixing between S1 and S2 as well as mixing between S2 and the atmosphere, shaded light gray. Error bars are not included with black circles for clarity. In other locations, if error bars are not visible, they are smaller than the data points.
Table 1: Overview of collected data. ‘Discrete’ measurements refer to the laboratory.

| Cave | State | Collection date | Sites measured | [CH₄] min (ppmv) | [CH₄] max (ppmv) | [CO₂] min (ppmv) | [CO₂] max (ppmv) | Methods |
|------|-------|-----------------|----------------|------------------|------------------|-----------------|-----------------|---------|
| 1    | AL    | 2015-04-07      | 1              | NA               | 2.2              | NA              | 378             | In-situ |
| 2    | IN    | 2013-08-10      | 4              | 0.42             | 1.89             | 2140            | 8000            | Discrete |
| 3    | IN    | 2013-08-07      | 3              | 0.23             | 4.2              | 13400           | 17900           | Discrete |
| 4    | IN    | 2013-06-18      | 2              | 0.3              | 0.7              | 3900            | 5200            | Discrete |
| 5    | IN    | 2013-06-29      | 3              | 0.4              | 1.18             | 3300            | 8600            | Discrete |
| 6    | IN    | 2013-06-14      | 3              | 0.32             | 1.32             | 750             | 3700            | Discrete |
| 7    | KY    | 2012-05-05      | 4              | 0.3              | 0.9              | 3500            | 8000            | Discrete |
| 8    | KY    | 2012-05-06      | 5              | 0.1              | 0.4              | 5200            | 6200            | In-situ |
| 9    | KY    | 2012-05-07      | 7              | 0.08             | 0.6              | 900             | 1500            | In-situ |
| 10   | KY    | 2013-07-18      | 1              | NA               | 1.79             | NA              | 920             | Discrete |
| 11   | KY    | 2013-07-18      | 1              | NA               | 0.91             | NA              | 2790            | Discrete |
| 12   | KY    | 2013-07-18      | 1              | NA               | 1.37             | NA              | 1790            | Discrete |
| 13   | KY    | 2015-04-10      | 4              | 2.3              | 2.6              | 349             | 390             | Mixed |
| 14   | KY    | 2015-04-10      | 3              | 1.82             | 2.33             | 351             | 390             | In-situ |
| 15   | KY    | 2015-04-10      | 2              | 0.9              | 1.8              | 440             | 486             | Mixed |
| 16   | OH    | 2015-04-10      | 1              | NA               | 1.95             | NA              | 940             | In-situ |
| 17   | PA    | 2013-07-15      | 5              | 1.4              | 1.9              | 1430            | 2400            | Mixed |
| 18   | PA    | 2013-07-15      | 4              | 0.4              | 1.1              | 760             | 4250            | Mixed |
| 19   | PA    | 2013-07-15      | 4              | 0.4              | 0.5              | 900             | 1400            | Mixed |
| 20   | PA    | 2013-07-16      | 3              | 0.4              | 1.0              | 3390            | 4240            | Mixed |
| 21   | TN    | 2013-07-18      | 3              | 0.5              | 1.25             | 2210            | 2430            | Mixed |
| 22   | TN    | 2013-07-19      | 3              | 2.6              | 3.5              | 490             | 1610            | Mixed |
| 23   | TN    | 2015-04-07      | 7              | 0.2              | 1.02             | 1100            | 1600            | Mixed |
| 24   | TN    | 2015-04-08      | 6              | 0.6              | 1.6              | 1100            | 1515            | Mixed |
| 25   | TN    | 2015-04-09      | 7              | 0.2              | 0.8              | 1430            | 2100            | Mixed |
| 26   | TN    | 2015-04-09      | 3              | 0.6              | 0.79             | 630             | 660             | Mixed |
| 27   | VA    | 2015-07-17      | 5              | 0.6              | 1.0              | 6300            | 14700           | Mixed |
| 28   | VA    | 2015-07-17      | 4              | 0.4              | 0.8              | 2480            | 7600            | Mixed |
| 29   | VA    | 2015-07-17      | 5              | 0.4              | 1.6              | 1100            | 8740            | Mixed |
| 30   | VA    | 2015-07-18      | 5              | 0.3              | 1.5              | 1090            | 19900           | Mixed |
| 31   | VA    | 2015-07-18      | 6              | 0.4              | 2.2              | 410             | 8900            | Mixed |
| 32   | NZc   | 2014-10-04      | 8              | 1.5              | 2.8              | 500             | 590             | In-situ |
| 33   | NZ    | 2014-10-04      | 3              | 1.0              | 1.7              | 500             | 620             | In-situ |
| 34   | NZ    | 2014-10-04      | 6              | 1.0              | 1.8              | 500             | 1000            | In-situ |
| 35   | IN    | 2014-06-11      | 2              | 0.14             | 0.3              | NA             | NA             | In-situ |
| 36   | MO    | 2016-09-24      | 9              | 0.1              | 1.5              | 2590            | 6190            | In-situ |

a Dates are formatted yyyy-mm-dd  
b NA = not applicable where only 1 sample was obtained.  
c NZ = New Zealand, d NA = Only CH₄ was measured
### Table 2: *In-situ* instrumentation used in this study.

| Instrument | Maker                  | Location         | Method                     | Analytes Measured | Lower detection limits |
|------------|------------------------|------------------|----------------------------|-------------------|------------------------|
| GasFinder2 | Boreal Laser           | Spruce Grove, Alberta, Canada | Tunable Diode Laser Spectroscopy Tunable Diode Laser | CH₄ | 1 ppmv m⁻¹ |
| Inspectra  | Gazomat, WEST Systems  | Pontedera, Italy  | Tunable Diode Laser Spectroscopy Tunable Diode Laser | CH₄ | 0.1 ppmv |
| LGD F200   | Axetris, SARAD          | Kägiswil, Switzerland | Tunable Diode Laser Spectroscopy Tunable Diode Laser | CH₄ | 0.2 ppmv |
| madIR-D01 CO2 | Madur, SARAD | Zgierz, Poland | Tunable Diode Laser Spectroscopy | CO₂ | 400 ppmv |
| DX4030     | Gasmet                  | Milton Keynes, United Kingdom | Fourier Transform Infrared Spectroscopy Non-dispersive Infrared | CH₄, CO₂, NH₃, | 0.3 ppmv CH₄, 200 ppmv CO₂, 0.1 ppbv NH₃ |
| LI820      | LICOR, WEST Systems     | Pontedera, Italy, Dresden, Germany | Spectroscopy Alpha spectroscopy | CO₂ | 5 ppmv |
| RTM 2200   | SARAD                   | Germany           |                             | Rn | 0 Bq/m³ |