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Subterranean microbial oxidation of atmospheric methane in cavernous tropical karst

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ABSTRACT

Subterranean methanotrophy is a potentially important but overlooked sink for the atmospheric greenhouse gas methane (CH_4). This study documents a microbial CH_4 sink in tropical subterranean karst cavities in Vietnam's northern karst province where porosity, steep topography, and scarce soil and vegetation cover foster the exchange of subterranean air with the atmosphere. Our data are based on (i) surveys of CH_4, carbon dioxide, and radon concentrations in the air of 11 caves, (ii) *in situ* mesocosm experiments in caves, as well as (iii) laboratory mesocosm measurements using sediment and rock from caves. The extent of CH_4 depletion in cave air depends on the ventilation rate and the availability of moisture to provide a habitat for CH_4-oxidizing bacteria, both of which are seasonally variable in northern Vietnam and in part depend on monsoonal activity. Mesocosm experiments using fresh *versus* sterilized rock and sediment confirmed the role of microbial methanotrophy towards uptake of CH_4 from cave air. Our results also suggest that within-cave heterogeneity of environmental variables like salinity may affect rates of CH_4 oxidation. We conservatively estimate that 150,000 metric tons of atmospheric CH_4 are microbially oxidized annually in the ~29,000 km^2 of Vietnamese tropical karst, which would compensate for ~7% of Vietnam's agricultural CH_4 emissions from rice farming and livestock. Future studies estimating the global fluxes of the atmospheric greenhouse gas CH_4 should consider subterranean karst as a potentially important CH_4 sink.

1. Introduction

Atmospheric methane (CH_4) is a potent greenhouse gas with globally and rapidly rising concentrations mainly due to anthropogenic activities (Etminan et al., 2016; Ciais et al., 2013; IPCC, 2013; U.S. EPA, 2010). Credible forecasting of global warming by climate models mandates knowledge of sources and sinks of atmospheric CH_4 (Xu et al., 2016). The two traditionally recognized major sinks for CH_4 are oxidation by (i) the hydroxyl radical in the upper atmosphere, and (ii) soil CH_4-oxidizing bacteria (MOB) found in soil and sediments (Naik et al., 2013; Dlugokencky et al., 2011). MOB habitats can also be found in other subterranean oxic environments with access to CH_4. Earth's continental surface includes ca. 10 to 20% of karst landforms (see p. 5 in Ford and Williams, 2007; Palmer, 1991) where caves and abundant smaller-size cavities and fractures provide abundant surface area and suitable habitat for MOB. The interconnected subterranean cavities in karst not only serve as drainage networks, but also provide conduits for air exchange with the atmosphere containing ~1.8 parts per million by volume (ppmv) CH_4 (Atkinson et al., 1983; Gregorič et al., 2014).

Several studies in temperate European, North American, and Australian regions have documented cases of CH_4 depletion in cave air relative to outside air (e.g., Waring et al., 2009; Mattey et al., 2013; Fernandez-Cortes et al., 2015; McDonough et al., 2016; Webster et al., 2016), but studies from tropical karst have been lacking. In contrast to karst in temperate climates, the exchange of subterranean air with the atmosphere in northern Vietnam's karst province and associated subterranean methanotrophy are fostered by the scarcity of soil and vegetation cover on the surface, a steep topography of abundant limestone outcrops, warm temperatures, and almost year-round availability of liquid water on many rock surfaces and in sediment. The fate of CH_4 in cave air is not only important from the perspective of a greenhouse gas sink, but CH_4 is also able to provide a carbon and energy source for

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heterotrophic subterranean biota (Jones and Macalady, 2016).

An assessment of CH_4 abundances and sinks in subterranean karst air is most easily accomplished in accessible caves, although the same biochemical and geochemical processes likely occur in smaller-size cavities that connect to the atmosphere. The seasonal dynamics and heterogeneity of trace gas compositions in cave air strongly depend on the rate and direction of ventilation, especially if a cave has multiple large openings for air access. This study used hydrogen-filled, neutrally buoyant balloons for accurate measurements of wind speed in cave passages. The ventilation of caves can be constrained by understanding the flux of carbon dioxide (CO_2) and radon in cave air. For example, a longer residence time of air in caves tends to decrease the CH_4 concentration, but often leads to accumulation of carbon dioxide and radon (e.g., Atkinson et al., 1983; Kowalczk and Froelich, 2010; Gregorič et al., 2014). None of the studied caves in northern Vietnam exhibited a sustained and strongly elevated radon concentration to plausibly explain low CH_4 concentrations *via* abiotic oxidation that is triggered by radioactivity in cave air (Fernandez-Cortes et al., 2015).

Few data have been published about rates of CH_4 oxidation in cave air. This study's data on rates of MOB activity from 11 caves add to previously available limited evidence from two caves on Cát Bà Island in coastal northern Vietnam (Lennon et al., 2017). In spite of substantial spatial and temporal variability of CH_4 uptake in cave environments, the available matrix of observations in, and CH_4 profiles along caves encouraged us to synoptically arrive at an overall rough estimate of CH_4 biodegradation in Vietnamese karst. Field and laboratory data on CH_4 depletion in contact with karst rock and sediment suggest that subterranean CH_4 oxidation in tropical karst represents a globally significant sink for atmospheric CH_4.

2. Materials and methods

2.1. Characterization of field sites

Karst occupies ~9% (i.e. ~29,000 km^2) of Vietnam's land surface and occurs in central and northern Vietnam (Trần and Nguyễn, 1986; Trần, 2005). The ~22,000 km^2 of northern Vietnamese karst represent ~19% of the northern region (Trần, 2005) within a tropical humid belt featuring typical tropical karst landforms, such as dolines, deeply incised valleys, and caves. The northern karst outcrops comprise mostly carbonates that are intercalated with minor terrigenous and/or marine siliceous members (Trần and Nguyễn, 1986; Do, 1998; Tran et al., 2013) and span from Early Cambrian to late Mesozoic ages. Regional tectonic activities variably caused thickening, fragmentation and dismemberment of rock units. It has been proposed that the development of the unique karst landscape in northern Vietnam resulted from a combination of deposition, deformation (Tran et al., 2013), and recent sedimentary and weathering processes in a tropical monsoon climate (Dang et al., 2009).

We conducted field work in 11 caves in May and November/December 2015 in (i) the mountainous karst in Vietnam's most northern Hà Giang province (Masschelein et al., 2007) and (ii) in low-altitude, coastal karst on Cát Bà Island (Fig. 1; Table 1). A main part of the northern Vietnamese mountainous karst area is the *Đồng Văn Karst Plateau Geopark* where some of the visited caves are located (GGN, 2010).

The monthly mean temperature and precipitation profiles for the city of Hà Giang in Hà Giang Province and for Cát Bà Island are shown as hythergraphs (Fig. 2; National Hydro-Meteorological Service of Vietnam, 2012). The monsoonal pattern of peak precipitation in summer with contemporaneously elevated temperatures is apparent for both regions, although Hà Giang tends to be slightly cooler than coastal areas and its precipitation is essentially symmetrically distributed around July as the wettest month, whereas precipitation on Cát Bà Island is left-skewed with more precipitation occurring in late summer. Seasonal monsoon rain provides for groundwater flow, whereas surface water flow is rare due to highly permeable karst rock with generally sparse soil cover.

2.2. In situ gas analyses in caves

Gas measurements in caves were performed in readily accessible locations where instruments could be positioned on level ground. Operating personnel stepped aside to avoid artifacts from human breath. When the entrances and passages of some caves proved to be too hazardous for transporting sensitive instruments, we organized teams to capture 200-L volumes of cave air in large plastic bags. The sampling of air avoided the influence of human breath. The bags were then rapidly carried to safe locations where gas samples were transferred into instruments with short segments of Tygon® plastic tubing. Concentrations of CH_4 and CO_2 in cave air were measured in May and Nov/Dec 2015 with a portable Gasmet DX-4030 FTIR analyzer (*Gasmet Technologies Oy*, Finland) that was calibrated daily with ultrapure nitrogen gas. The precision of measured gas concentrations is ± 0.3 ppmv for CH_4 and ± 30 ppmv for CO_2 (± 95% confidence intervals). Additional CH_4 and CO_2 measurements were performed with a SARAD® RTM 2200 instrument (*SARAD®*, Dresden, Germany, with an Axetris® laser OEM Module LGC F200 CH_4 detector, *Axetris® AG*, Switzerland) that had been calibrated against the Gasmet DX-4030 FTIR analyzer. With detailed data from Nà Luông, Rồng, and Pải Lủng caves, we were able to map CH_4 and CO_2 concentrations by gridding unevenly distributed data with adjusted-tension continuous curvature splines using the surface module of Generic Mapping Tools (Wessel et al., 2013) with a tension factor 0.25, convergence limit 0.01, and 1.5 m distance between estimated points.

Radiation from the decay of radon (^{222}Rn) in caves was α-spectroscopically quantified with the portable SARAD® RTM 2200 instrument. We did not encounter high CO_2 concentrations that would have limited the precision of the SARAD® RTM 2200 (Shahrokhi et al., 2015).

Wind speed in caves was measured by the motion of free-floating hydrogen-filled latex balloons. Hydrogen gas was generated on-site in a 1-L bottle that was half filled with water and received ~3 g of calcium metal turnings wrapped in tissue paper. A deflated latex balloon was connected to the mouth of the bottle and became inflated as elemental hydrogen gas developed pressure. Inflated balloons were tied with short strings and ballasted with adhesive tape to neutral buoyancy before being released in cave passages.

2.3. Mesocosm experiments

We performed mesocosm experiments to test for methanotrophy in a cave (using cave rocks) and in the laboratory in Hanoi (using 10 sediments and 1 rock sample from 5 different caves; Table 2). Rocks weighing from 0.1 to 1 kg were collected in Minh Châu cave, whereas fine-grained, clay-rich sediments from cave floors were shoveled into polyethylene bags. Sediments were not allowed to lose moisture prior to their use in mesocosm experiments in the laboratory in Hanoi within a few days after transport. In contrast to our earlier mesocosm experiments in Minh Châu cave that had used single-layer polyethylene bags (Lennon et al., 2017), in this study we attempted to minimize diffusive loss of CH_4 through the 0.0475 mm thick polyethylene bags by nesting one bag within another (i.e. 'double-bags'). We conducted *in situ* double-bag control experiments in Minh Châu cave by spiking 200 L of air with a few milliliters of CH_4 from biogas (~90 vol% CH_4) and quantifying the diffusive loss of CH_4 over ~13 to 14 h. Biogas was obtained from a farmer's fermentation plant that was operated with organic farm waste near Hanoi. The 'sweet' smell of CH_4-rich biogas proved the absence of hydrogen sulfide. In contrast to industrially generated CH_4, biogas does not contain acetylene, which can deactivate methanotrophy (Pham et al., 2015). Some mesocosm experiments were conducted in Minh Châu cave where overnight deployment of polyethylene double-bags with local rocks tested for patterns of *in situ*

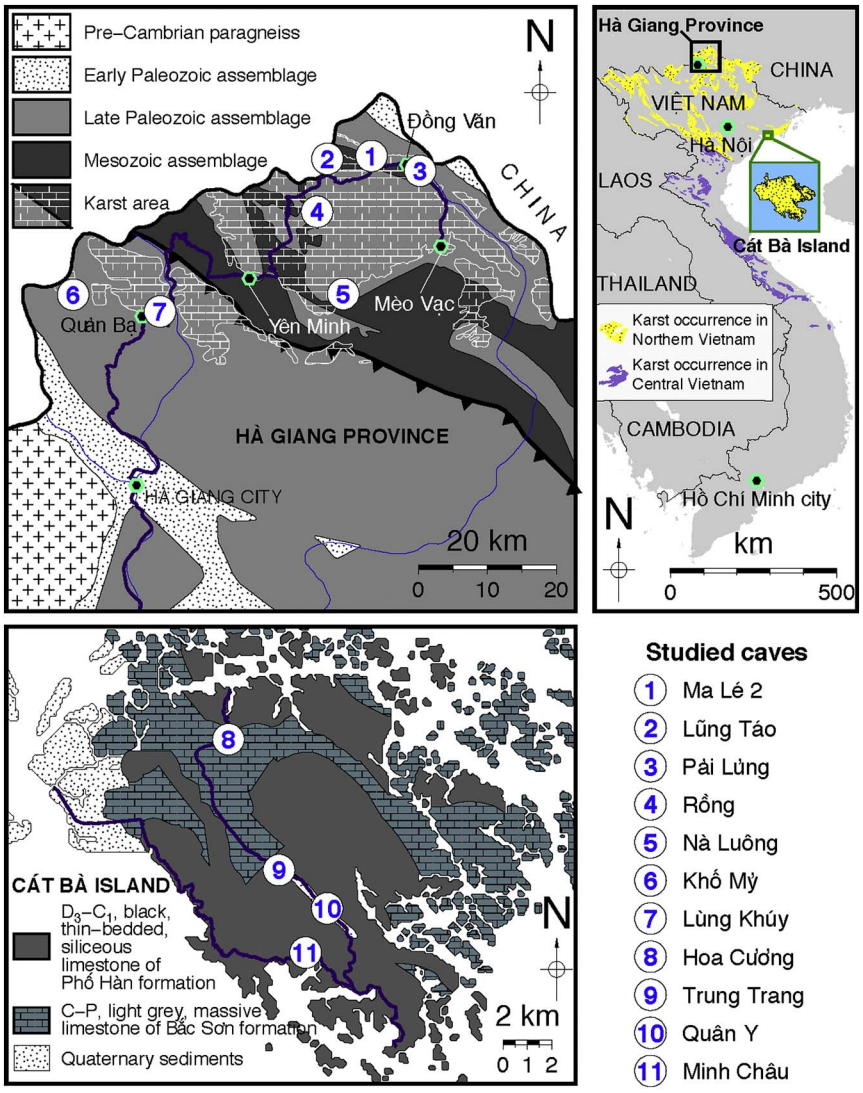

Fig. 1. Locations of 11 studied caves in Hà Giang province and on Cát Bà Island in northern Vietnam. The customized map is based on Trần (2005) and Tran et al. (2013). Further details on cave locations are in Table 1.

microbial CH_4 oxidation. We initiated the mesocosm experiments by misting the interior of the inner polyethylene bag with ~20 mL water after adding rocks or sediment from caves. Experiments with sterilized rocks in Minh Châu cave used 10 wt% aqueous sodium hypochlorite bleach solution that was sprayed onto rocks prior to deployment in mesocosms. We determined the concentration of gasses at the

Table 1
Characteristics of 11 northern Vietnamese caves in the order of decreasing latitude that were measured in May 2015 and/or in November/December 2015. Air flow was measured in narrow cave passages with maximum wind speed.

Cave number and name	Latitude (degrees)	Longitude (degrees)	Altitude (m)	Temperature in cave (°C)		Max. wind speed in cave ($m\ s^{-1}$)		^{222}Rn in cave air (in) and outside air (out) ($Bq\ m^{-3}$)			
				May	Nov/Dec	May	Nov/Dec	May		Nov/Dec	
								In	Out	In	Out
1) Ma Lé 2	23.290477	105.313094	1055	n.d.	17	n.d.	0	n.d.	n.d.	n.d.	n.d.
2) Lũng Táo	23.286466	105.251212	1555	n.d.	12	n.d.	0.13	n.d.	n.d.	0	0
3) Pải Lủng	23.269672	105.381418	1043	22	18	0.02	0	50	2800	0	0
4) Rồng	23.212077	105.236597	1440	21	14	0.01	0.03	50	5500	60	210[a]
5) Nà Luông	23.096527	105.273548	251	22	15	0.01	0	405	520	n.d.	n.d.
6) Khố Mỷ	23.095477	104.893818	954	21	n.d.	0	n.d.	50	457	n.d.	n.d.
7) Lùng Khuý	23.071697	105.016030	751	n.d.	18	n.d.	0.09	103	102	0	0
8) Hoa Cương	20.837227	106.982436	19	24	n.d.	0.02	n.d.	75	75	n.d.	n.d.
9) Trung Trang	20.785780	107.002308	33	24	n.d.	n.d.	n.d.	n.d.	62	n.d.	n.d.
10) Quân Y	20.770662	107.021274	42	25	n.d.	n.d.	n.d.	n.d.	60	n.d.	n.d.
11) Minh Châu	20.753282	107.012797	17	24	22	0.05	0.06	0	115	0	0

n.d. = not determined.
[a] Repeat radon (^{222}Rn) measurements of up to 450 $Bq\ m^{-3}$ in the air of Rồng cave in December 2016 qualitatively confirmed observations from December 2015.

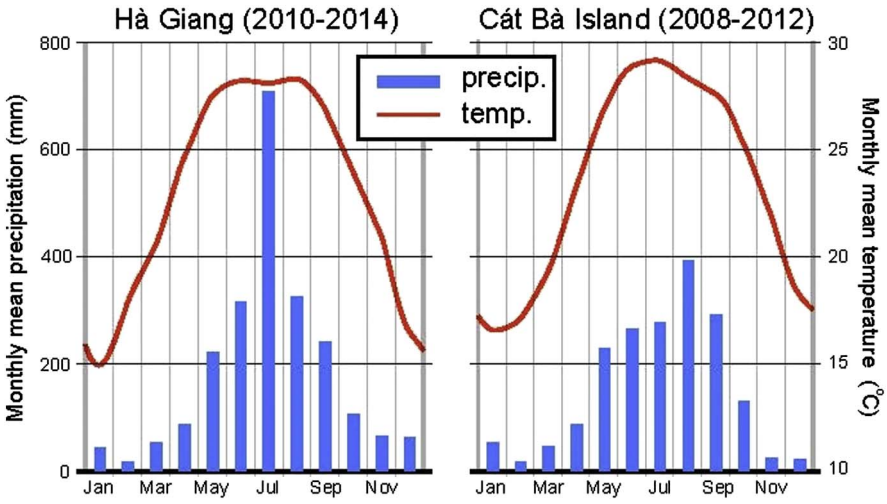

Fig. 2. Hythergraphs showing monthly mean precipitation and temperature profiles based on 2010–2014 data from Hà Giang and 2008–2012 data from Cát Bà Island (National Hydro-Meteorological Service of Vietnam, 2012).

beginning and end of experiment which lasted 14 h. Atmospheric pressure and temperature were recorded during times of measurements for calculating CH_4 fluxes. The analytical precision of CH_4 measurements for several mesocosm experiments was enhanced by raising CH_4 concentrations up to 50 ppmv by adding ~2 to 5 mL CH_4 from a lecture bottle with biogas into double-bags. We note that artificially elevated CH_4 abundances in mesocosm experiments may induce artifacts in MOB activity. Our experiments were designed to principally test for MOB activity *via* the rate of decline of CH_4 abundances. Closed chamber flux measurements occasionally express non-linear behavior over time, but the methodological and analytical uncertainties are generally smaller than the environmental variability in the field (Pirk et al., 2016).

2.4. Laboratory experiments

Because it was impractical to perform multiple mesocosm experiments lasting several days each in remote caves, we estimated CH_4 consumption by conducting double-bag experiments under laboratory conditions. We used the same type of polyethylene bags, ~200 L of ambient air with an atmospheric abundance of ~2 ppmv CH_4, various levels of initial enrichments with CH_4 from biogas, ~0.5 to ~2 kg of rock or sediment that was from cave floors (note that the amount of sediment/rock used for *in situ* mesocosm experiments in caves was much larger at ~10 kg), and a volume of 20 mL of water sprayed into bags to provide moisture. After performing experiments with 'live' sediments and rocks in the laboratory, we sterilized the sediments and rocks for 30 min at 150 °C in a pressure cooker, let the cooker cool, and re-deployed the sterilized sediments and rocks in follow-up 'dead' double-bag mesocosm experiments. Some plastic bags were connected *via* pairs of Tygon® tubing for outflow and return flow with the SARAD® RTM 2200 in gas reflux mode for continuous measurements overnight. Most bags were measured only initially and in subsequent ~12 h increments over a total time period of ~80 h for 'live' experiments and ~18 to 38 h for 'dead' experiments.

2.5. Determination of chlorine contents in cave sediments

Soluble chlorine in dry sediment served as a proxy for salinity in sediment. Chlorine was quantified in the Environmental Analytical Laboratory at VNU University of Science (HUS, Hanoi) according to a modified protocol No. TCVN 8727-2012. In brief, dry soil with a grain size below 2 mm was added to deionized water at a weight ratio of 1 to 5. After stirring at room temperature, the suspension was filtered and the filtrate was reacted at room temperature with 3 mL ~15 wt% aqueous hydrogen peroxide H_2O_2 solution for 1 h. A known volumetric aliquot was adjusted with H_2SO_4 or NaOH to circumneutral pH,

Table 2
Net methane (CH_4) oxidation rates (i.e. overall CH_4 loss minus a diffusional loss of CH_4 through double-bags at a rate of 0.01 ppmv h^{-1}) observed in (A) laboratory experiments in Hanoi and (B) average values from 6 mesocosm experiments in Minh Châu cave. Each double-bag contained either 'live' or 'dead' (i.e. autoclaved) sediment or rock from one of 5 caves. 'H_2O in sediment' refers to the original water content in sediment from cave floors. The chlorine (Cl) concentration refers to water-soluble chloride in dry sediment.

Bag #	Cave, location of origin of rocks, sediment (sed.)	Decline of CH_4 (ppmv h^{-1})				Sediment or rock in bags (kg)	Oxidation of CH_4		H_2O in sediment (wt%)	Cl in dry sed. (mg kg^{-1})
		'Live' bags			'Dead' bags					
		Min.	Max.	Mean	Mean		(ppmv h^{-1} kg^{-1})	(ng h^{-1} kg^{-1})		
(A) *Mesocosm experiments in the laboratory in Hanoi*										
1	Nà Luông #2, sed.	0.00	0.27	0.03	0.01	0.65	0.04	5.2	28.3	75
2	Nà Luông #3, sed.	0.12	0.20	0.16	0.01	0.60	0.24	31.5	31.8	51
3	Nà Luông #1, sed.	0.06	0.75	0.37	0.01	0.60	0.60	78.7	29.6	45
4	Pải Lủng #1, sed.	0.02	0.09	0.04	0.01	0.75	0.04	5.2	17.1	32
5	Ma Lé 2 #2, sed.	0.02	0.09	0.04	0.01	0.65	0.05	6.6	24.5	57
6	Lùng Khúy, sed.	0.01	0.08	0.03	0.01	0.55	0.04	5.2	30.7	36
7	Ma Lé 2 #1, sed.	0.01	0.08	0.04	0.01	2.35	0.01	1.3	27.1	54
8	Minh Châu, sed.	0.06	0.16	0.10	0.01	0.90	0.10	13.1	n.d.	n.d.
9	Pải Lủng #3, sed.	0.01	0.06	0.03	0.02	0.90	0.01	1.3	38.5	294
10	Pải Lủng #2, sed.	0.01	0.07	0.04	0.00	0.80	0.04	5.2	27.9	58
11	Minh Châu, rock	0.03	0.16	0.10	0.01	1.05	0.09	11.8	n.d.	n.d.
(B) *Mesocosm experiments in Minh Châu cave*										
12	Minh Châu, rock	0.09	0.37	0.21	0.00	10.00	0.02	2.6	n.d.	n.d.

n.d. = not determined.

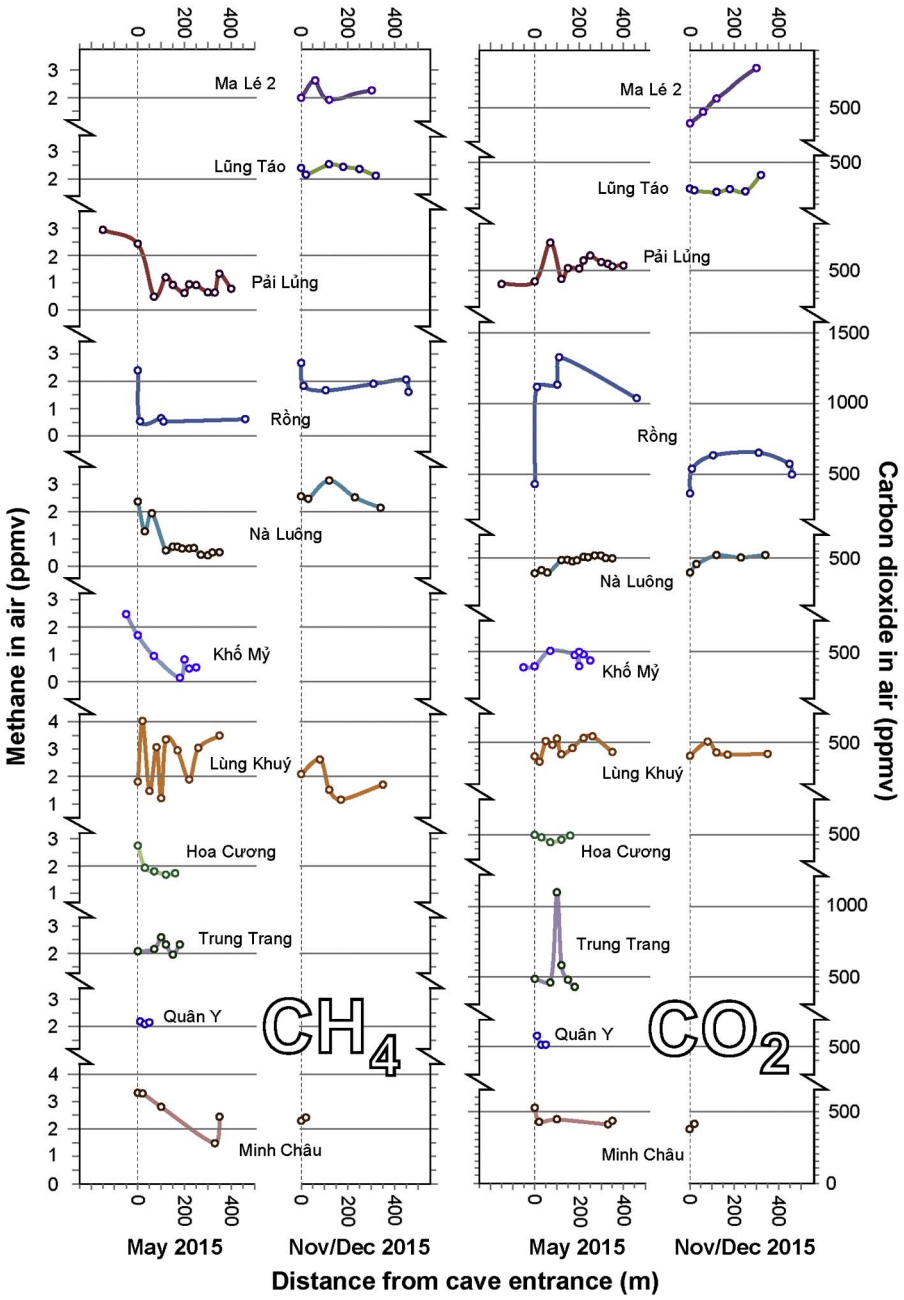

Fig. 3. Methane (CH_4) and carbon dioxide (CO_2) concentrations in northern Vietnamese caves as a function of distance from main cave entrances for two seasons. Caves are ordered sequentially from north to south as in Fig. 1 and Table 1. The same CH_4 and CO_2 data for Nà Luông, Rồng, and Pải Lủng caves are plotted as concentration patterns on respective cave maps in Fig. 4. The spacing of tick marks along the left and right Y-axes uniformly expresses 0.5 and 50 ppmv increments, respectively.

followed by titration with 0.02 N $AgNO_3$ solution. The endpoint was indicated by the formation of silver chromate in the presence of potassium chromate K_2CrO_4.

3. Results

3.1. In situ cave surveys

Transects of CH_4 and CO_2 concentrations from cave entrances to interior sampling locations often indicated a depletion of CH_4 and a rise in CO_2 (Fig. 3). In addition, we observed strong seasonal differences in the concentrations of CO_2 and CH_4. Within a cave, the composition of air in well ventilated caves was strongly influenced by outside air, for example in Quân Y and Trung Trang caves. With the exception of the extremely well ventilated Minh Châu cave at ~0.5 m s^{-1} air flow between a natural and an artificial emergency exit, all observed wind speeds were below 0.15 m s^{-1} (Table 1). With the exception of Rồng Cave where in one location 5500 Bq m^{-3} were reached in May 2015, none of the studied Vietnamese caves featured rooms or passages with radioactivity from radon exceeding 3000 Bq m^{-3}, and most caves measured radon below 500 Bq m^{-3} (Table 1). Three caves were surveyed with sufficient resolution to map CH_4 and CO_2 abundance patterns for interpreting the distribution and dynamics of trace gases in cave air (Fig. 4). An overall plot of individually measured concentrations of CH_4 *versus* CO_2 in cave air (Fig. 5) demonstrates that cave air is typically enriched in CO_2 relative to outside air. CH_4 concentrations in cave air are generally below those of outside air measured in front of caves, and are lower than CO_2 concentrations by more than two orders of magnitude.

3.2. Mesocosm experiments in caves

The rate of CH_4 oxidation was determined in mesocosm experiments. The diffusive loss of CH_4 through polyethylene double-bags was subtracted from the overall CH_4 loss in mesocosm experiments with cave rock or sediment in double-bags to arrive at the net rate of

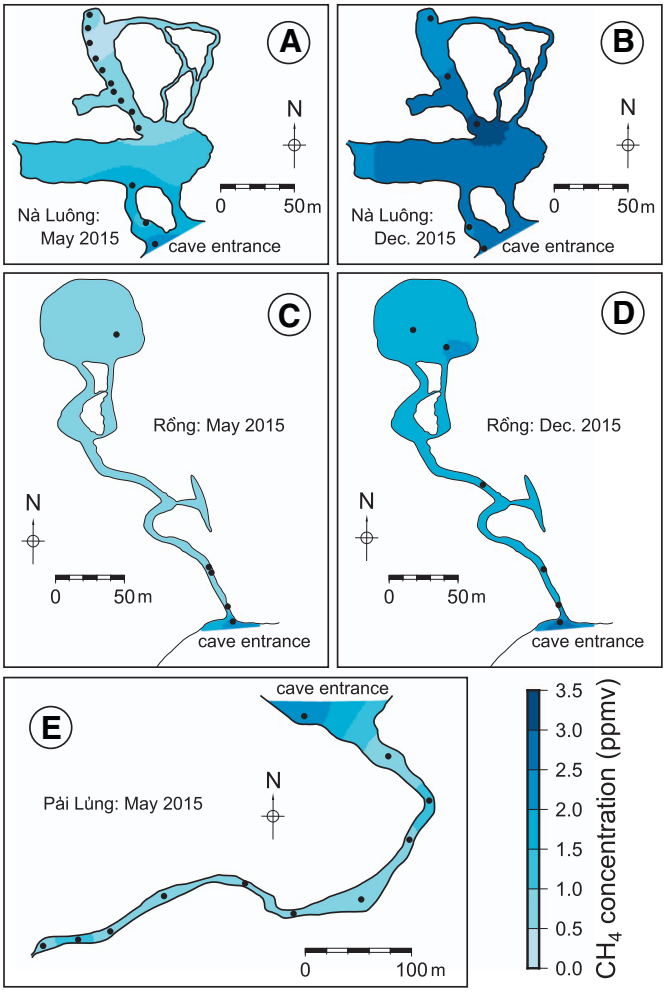

Fig. 4. Distribution of methane (CH_4) concentrations in the air of Nà Luông, Rồng, and Pải Lủng caves in May, November and December 2015. Black dots indicate sampling stations. Unevenly distributed data were gridded with adjusted tension continuous curvature splines using the surface module of Generic Mapping Tools (Wessel et al., 2013). Maps of Nà Luông and Pải Lủng caves were adopted from Masschelein et al. (2007).

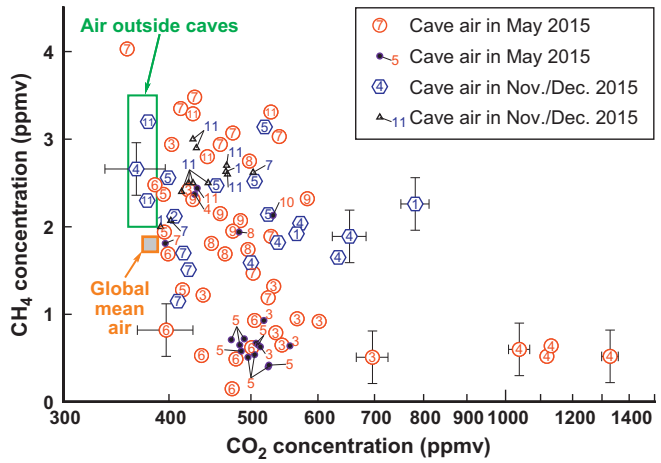

Fig. 5. Methane (CH_4) *versus* carbon dioxide (CO_2) concentrations measured in northern Vietnamese caves. The numbering of caves is defined in Table 1. CH_4 concentrations in air outside of cave entrances (range identified by rectangle) in excess of the global atmospheric average of ~1.8 ppmv (identified by square) may be due to agricultural methanogenesis and leakage from domestic biogas reactors. The analytical precision of ± 0.3 ppmv for CH_4 and ± 30 ppmv for CO_2 (± 95% confidence intervals) refers to the G-ASMET DX-4030 FTIR analyzer. Shown select error bars are representative for all data.

methanotrophic CH_4 consumption. *In situ* control experiments in Minh Châu cave with double-bags containing air with various levels of CH_4-enrichment quantified the diffusive CH_4 loss over ~13 to 14 h (Fig. 6). Although the rate of CH_4 loss to the outside atmosphere *via* diffusion through polyethylene bags should be proportional to the gradient along CH_4 partial pressures, the use of double-bags in 'dead' mesocosm experiments instead of single bags greatly reduced the diffusive CH_4 loss. The average diffusive CH_4 loss of 0.01 ppmv h^{-1} (i.e. 1.3 ng h^{-1}) was used as a correction for 200-L mesocosm experiments with 'live' rock or sediment.

The average rate of CH_4 oxidation by methanotrophs associated with 'live' cave rocks was 2.6 ng h^{-1} kg^{-1} (i.e. 0.02 ppmv h^{-1} kg^{-1}; range from 1.3 to 5.2 ng h^{-1} kg^{-1}). In comparison, the CH_4 microbial oxidation rates in Hoa Cương and Minh Châu caves had previously been estimated with 'live' cave rocks in single-layer polyethylene mesocosm experiments to range from 0.5 to 1.8 ng h^{-1} kg^{-1} (Lennon et al., 2017). However, the earlier mesocosm experiments had relied on regular air without CH_4 enrichment. The significant depletion of CH_4 during the course of the earlier experiments may have 'starved' the MOB and resulted in decreased rates of MOB activity.

3.3. Laboratory experiments

CH_4 oxidation rates in mesocosm experiments were lower when sediment or rock was used from Ma Lé 2, Pải Lủng, and Lùng Khúy caves relative to Nà Luông and Minh Châu caves (Fig. 7). The highest mean CH_4 oxidation rate was found for 'live' sediment #1 from Nà Luông cave at 78.7 ng h^{-1} kg^{-1}. This result cannot be due to leakage from the double-bag, because the final CH_4 concentration in the bag was lower than the CH_4 concentration in ambient Hanoi air outside of the mesocosm. The lowest rates for 'live' sediment of 1.3 ng h^{-1} kg^{-1} were encountered for samples Ma Lé 2 # 1 and Pải Lủng # 3, and were similar to the typical diffusive loss of CH_4 from 'dead' double-bags (Fig. 7).

The average overall CH_4 loss rate from 11 'live' laboratory mesocosm experiments was 11.7 ng h^{-1} (range 4.0 to 48.7 ng h^{-1}; i.e. 0.09 ppmv h^{-1} within a range of 0.03 to 0.37 ppmv h^{-1}; Fig. 8). In comparison, the average overall CH_4 loss rate from 11 'dead' laboratory mesocosm double-bag experiments was 1.3 ng h^{-1} (range 0.0 to 2.6 ng h^{-1}; i.e. 0.01 ppmv h^{-1} within a range of 0.00 to 0.02 ppmv h^{-1}; Table 2). The rate of microbial CH_4 oxidation was estimated for each 'live' double-bag experiment by subtracting an average diffusive CH_4 loss of 1.3 ng h^{-1} (i.e. 0.01 ppmv h^{-1}) from the overall CH_4 loss.

4. Discussion

Our findings suggest that CH_4 oxidizing bacteria (MOB) represent a potentially large CH_4 sink in terrestrial subterranean ecosystems. MOB activity in porosity spanning from soils (Fest et al., 2017) to caves is gaining global relevance by our finding of MOB activity in tropical karst where abundant and well ventilated porosity offers a year-round habitat for MOB. Our multi-seasonal *in situ* and laboratory kinetic measurements and empirically determined CH_4 concentration gradients along caves allow a first overall estimate of CH_4 oxidation in tropical karst as exemplified in northern Vietnam.

4.1. Seasonality and monsoon affecting ventilation and moisture availability in caves

The tropical warmth in Vietnamese caves and monsoonal precipitation exceeding 3500 mm yr^{-1} (National Hydro-Meteorological Service of Vietnam, 2012; Fig. 2) stand in stark contrast to most caves at higher latitudes in temperate climates. Seasonal temperature differences between outside air and subterranean air forces air circulation in Vietnamese caves that is often enhanced by wind generating air

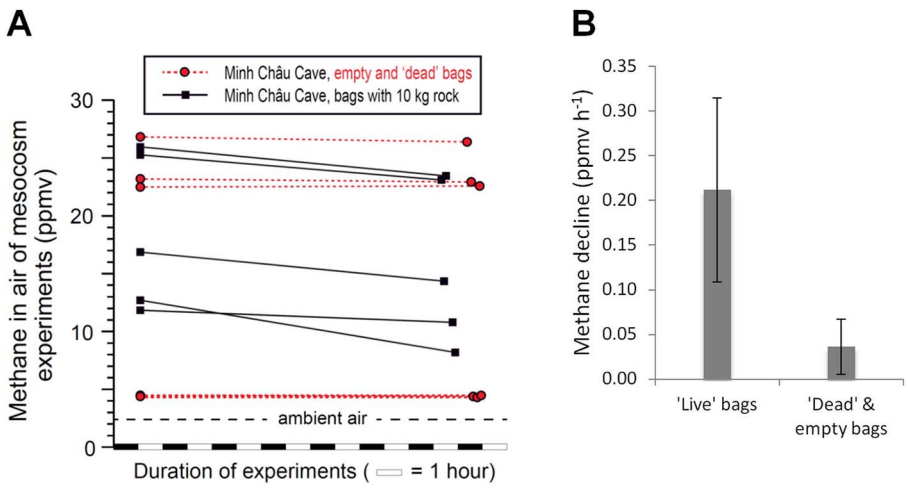

Fig. 6. (A) CH_4 oxidation rate expressed in terms of changes in CH_4 concentrations in mesocosms (200-L polyethylene double-bags) over ~13 to 14 h in Minh Châu cave. Each white or dark segment of the X-axis represents 1 h. All double-bag experiments had received additional CH_4 from biogas to elevate initial CH_4 concentrations above the natural CH_4 concentration in ambient air in Minh Châu cave of ~2.4 ppmv in order to increase the analytical precision. Dashed lines refer to control empty and 'dead' bags (i.e. with sterilized cave rocks) that lost CH_4 due to diffusion of CH_4 through polyethylene bags. In contrast, solid lines indicate bags with ~10-kg aliquots of local 'live' rocks from the cave that likely harbored methanotrophic microbes. Differences in CH_4 loss (i.e. enhanced negative slope of lines) are interpreted in terms of microbial methanotrophy. (B) Average rates of CH_4 oxidation in 200-L polyethylene double-bags that either contained ~10-kg aliquots of local 'live' or 'dead' rocks from Minh Châu cave, or contained no rocks. Each of the two categories included measurements from 5 to 9 double-bag experiments over ~13 to 14 h in the cave. Bars display average values, whereas brackets indicate standard deviations.

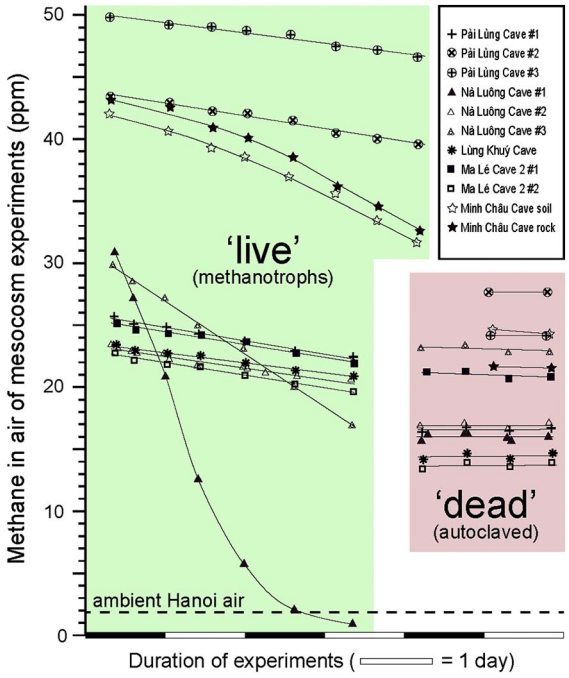

Fig. 7. Laboratory experiments measuring the loss of CH_4 from air in polyethylene double-bag mesocosms over several days. Ten sediment samples and one rock sample (~0.5 to ~2 kg each; Table 2) from five caves were placed into double-bags. The 200 L of air in each bag were spiked with various amounts of CH_4 from biogas to increase the analytical precision of the SARAD® RTM 2200 in closed-circuit reflux mode. The initial 'live' experiments used unaltered samples harboring microbial methanotrophs. The same sediment and rock samples were subsequently autoclaved and used again in 'dead' control experiments. Differences in CH_4 loss (i.e. negative slope of lines) between live and dead experiments are interpreted in terms of microbial methanotrophy.

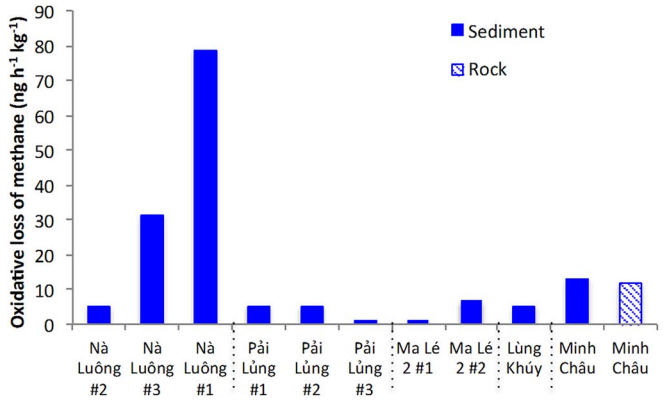

Fig. 8. Rates of CH_4 oxidation in laboratory experiments. Filled bars represent experiments with 'live' sediments from caves, whereas hachure indicates a 'live' mesocosm experiment with rock from Minh Châu cave.

pressure gradients across the steep geomorphology. Multiple visits to individual caves provided evidence for strong seasonal changes in temperature gradients influencing the direction and speed of air exchange. By spring and early summer, warmer and more humid air enters relatively cooler caves and condenses moisture on surfaces that are not wetted by occasional drip water from above. An extreme example of seasonal condensation is shown for the Quân Y hospital cave in the south of Cát Bà Island near Cát Bà town (Fig. 1) where a former hospital concrete structure inside a strongly ventilated natural cave is glistening with moisture in June 2015, shortly after cold cave air began to be replaced by warm and moist air during summer (Fig. 9). Seasonal condensation also affects natural rock surfaces in caves. The availability of condensation and drip water generates a suitable habitat for microbial activity in Vietnam's tropical caves.

4.2. Methanotrophy in ventilated caves causes oxidation of CH_4 in cave air

Local agricultural and natural methanogenesis seems to have increased the CH_4 concentration in air outside of many cave entrances significantly above the global atmospheric average of ~1.8 ppmv (Fig. 3). Elevated CH_4 concentrations in air outside of cave entrances are likely due to local agricultural methanogenesis and leakage from domestic biogas reactors. A study of CH_4 in the air above a rice field near Hanoi documented CH_4 concentrations that could exceed 3.5 ppmv (Sandin, 2005). Southeast Asian agricultural areas are global hotspots for agricultural CH_4 emissions. The Global Methane Initiative (2010) reports that Vietnam's estimated anthropogenic CH_4 emissions ranked 20th in the world based on 2005 data, with rice cultivation accounting for the country's largest source of CH_4 emissions.

CH_4 concentrations in cave air tended to decrease with increasing residence time of air in caves. Our CH_4 concentrations and gradients in the air of tropical Vietnamese caves are qualitatively compatible with data from European and North American temperate caves, albeit the literature offers contrasting conclusions about the importance of MOB. For example, Fernandez-Cortes et al. (2015) suggested that MOB contribute minimally to rates of CH_4 oxidation in Spanish caves with poor ventilation where temperatures range from 10.8 to 17 °C and local annual rainfall is below 1400 mm yr^{-1}. It has been established that CH_4 can be used as a carbon and energy source by microorganisms in

Fig. 9. Abandoned concrete hospital structure in Quân Y Hospital Cave on Cát Bà Island near Cát Bà town. The hospital structure had been built inside of a natural, well ventilated cave and offers visual evidence for seasonal condensation of moisture. Dry conditions in December 2015 (left) contrast with condensation from humid air in June 2015 (right).

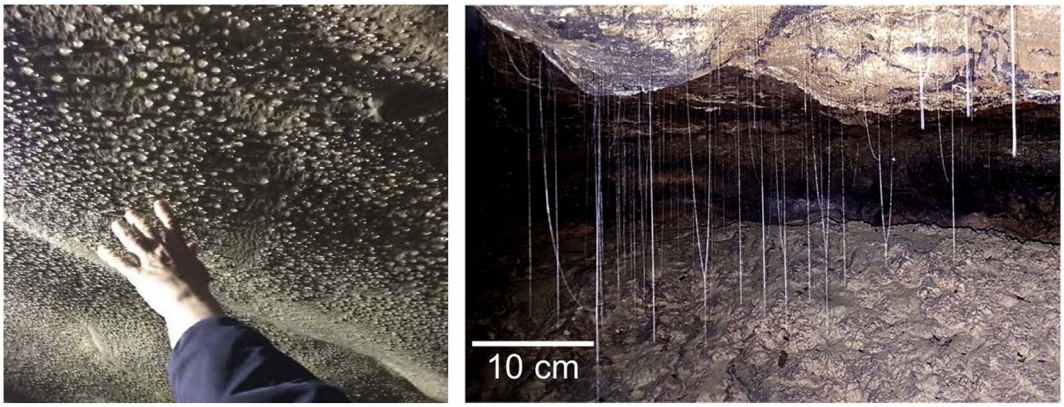

Fig. 10. Evidence for possible microbial habitats in caves: *Left*: beading of condensed water on the uneven limestone ceiling of Minh Châu cave in May 2015 suggests the presence of a hydrophobic microbial film. Hydrophobicity may enable methanotrophs to maintain unobstructed access to CH_4 in air. *Right*: 'slime threads' descending from dry surfaces in Rồng cave in November 2015.

suitable subterranean moist habitats (Jones and Macalady, 2016). The optimum temperature for methanotrophy is ~25 °C (King and Adamsen, 1992; Sommerfeld et al., 1993; Sitaula et al., 1995), although Le Mer and Roger (2001) found no significant temperature-related differences between 1 and 30 °C. Our earlier DNA-based evidence for the presence of methanotrophs in Vietnamese caves (Lennon et al., 2017) and this study's extensive evidence from mesocosm experiments for rapid CH_4 consumption by cave rock and sediment from cave floors (except after sterilization) unambiguously point to the important role of MOB in the oxidation of CH_4 in tropical subterranean karst environments.

It has been claimed that high levels of radioactivity from radon on the order of thousands to tens of thousands Bq m^{-3} can trigger ionization of air and entail the rapid oxidation of CH_4 in Spanish caves without the assistance of MOB (Fernandez-Cortes et al., 2015). All studied Vietnamese caves featured radioactivity far below the levels encountered in Spanish caves, and most caves measured radon below 500 Bq m^{-3} (Table 1). In the absence of a radiolytically triggered CH_4 oxidation mechanism in Vietnamese caves, the significantly declining CH_4 concentrations in overnight mesocosm experiments in 3 caves (including data in Lennon et al., 2017) with mostly low radon abundances ($\leq$ 100 Bq m^{-3}) using *in situ* limestone rocks underscored the ability of MOB in Vietnam's caves to consume CH_4 in cave air within a few hours, sometimes to levels below detection limit. Our data suggest that bacterial methanotrophy is sufficient to account for oxidative CH_4 loss in Vietnam's subterraneous karst cavities.

4.3. Spatially and temporally heterogeneous methanotrophy in cave sediment and rock

Soluble electrolytes in cave sediment and on rock surfaces can be imported by flying insects and bats. Salts can persist and accumulate in locations that are not occasionally washed by drip water or flowing water. Locally elevated salinity influences microbial habitats (Morrissey and Franklin, 2015). The highest measured chloride content in sediment at location #3 in Pải Lủng cave coincides with the lowest observed methanotrophic activity of any measured sediment (Table 2).

The putrid odor of some rocks and sediments after sterilization in a pressure cooker was likely caused by contamination with bat feces and urine. Nà Luông cave's large bat population locally deposits bat guano that is being used by local villagers as fertilizer for culturing orchids. Rocks from different parts of Nà Luông cave expressed strongly contrasting methanotrophic activities, which is presumably due to a localized influence from bat roosts (Fig. 8). Strong seasonal differences in cave CH_4 abundances in Nà Luông cave are best exemplified between May and December 2015 (Figs. 4A–B, 5). Methanotrophy was likely enhanced when cave rock surfaces were moist from condensation and drip water during May, whereas a stronger temperature gradient between dry outside air and relatively warmer cave air in December limited moisture availability. The single December measurement of an elevated CH_4 concentration in excess of the outside atmospheric CH_4 concentration in Nà Luông cave may be due to microbial methanogenesis in seasonal accumulations of bat feces.

A qualitatively similar, yet less pronounced seasonal difference in CH_4 abundance is apparent for Rồng cave (Fig. 4C, D) where relatively low radon and CO_2 abundances in December 2015 (also confirmed in

December 2016 when radon and CO_2 measured up to ~450 Bq m^{-3} and ~1000 ppmv) argue for enhanced ventilation in winter in combination with reduced methanotrophy relative to wetter conditions in parts of the cave in May. For both Nà Luông and Rồng caves, intermittently elevated CH_4 abundances cannot be explained by a deep geologic source of CH_4 and CO_2 outgassing from shale or other organic-rich sediments below the limestone, because CH_4 anomalies in December were not accompanied by elevated CO_2 concentrations (Fig. 3). Only in Pải Lủng cave, an intermittent maximum in CH_4 concentration ~200 m into the cave (Fig. 4E) coincides with a distinct peak of CO_2 (Fig. 3) and may have resulted from deep outgassing.

4.4. Evidence for possible microbial habitats in caves

During condensation of moisture on cave rocks in May 2015 we observed that only 'water-washed' walls and ceilings in well ventilated caves featured hydrophobic surfaces where water droplets were beading (Fig. 10), presumably as a result of the hydrophobic nature of a microbial film on the rock surface. We hypothesize that MOB form a hydrophobic film on rocks to prevent flooding of the rock surface and to maintain unrestricted access to CH_4-containing air. Bacterial consortia have been documented to form mixed-species biofilms on sedimentary rock surfaces (e.g., Włodarczyk et al., 2017). A different strategy to effectively filter CH_4 from passing air may be employed by microbes along 'slime threads' descending from parts of the ceiling in Rồng cave in November 2015 (Fig. 10). Similar slime threads are being produced as snares by carnivorous 'glow-worm' larvae of fungus gnats, for example in Waitomo Caves in New Zealand (The Encyclopedia of New Zealand, 2017). However, we could not discern any spiders, insects or their larvae in connection with 'slime threads' in Rồng cave.

4.5. Estimate of annual CH_4 oxidation in Vietnamese karst

In spite of the significant natural variability of methanotrophy in vadose environments both geographically and over time, the available data from northern Vietnam allow a first estimate of annual atmospheric CH_4 oxidation in regional tropical karst. Methanotrophs find a suitable habitat in porous limestone not only in caves (Tomczyk-Żak and Zielenkiewicz, 2016) but also in smaller-size fractures, cavities and general porosity in karst wherever air exchange with the atmosphere provides a supply of CH_4.

Our fieldwork did not generate data from the coldest (January) and warmest (July; Fig. 2) months of the year when ventilation is likely maximized due to highest temperature differences between cave air and outside air (Breitenbach et al., 2015). Therefore the re-supply rate of atmospheric CH_4 into karst and the observed CH_4 oxidation rates in caves during our field work were not at their peak, and our measured data can support a conservative estimate of methanotrophy in Vietnamese karst. The basis for our estimate is our observation from mesocosms that air with 2 ppmv CH_4 in contact with average moist 'live' cave rock or sediment loses most of its CH_4 in one day, in spite of the fact that the ratio of rock/sediment surface *versus* air is much lower than in subterranean cavities. This argues that the kinetics of methanotrophic CH_4 uptake in natural subterranean environments are faster than in our mesocosms. CH_4 concentrations outside of caves were often in excess of 2 ppmv due to regional agricultural emissions and leakage from residential biogas reactors (Fig. 5). As a consequence, our assumption of 2 ppmv CH_4 in outside air is a conservative basis for estimating the flux of CH_4 into caves and the subsequent subterranean oxidation of atmospheric CH_4.

The karst coverage in northern Vietnam is about 22,000 km^2 (Fig. 1). The average volume of connected porosity in near-surface, highly weathered limestone is about 20 vol% (Freeze and Cherry, 1979; Ford and Williams, 2007). As a first approximation we assume that outside air with ~2 ppmv CH_4 content daily exchanges with subterranean air down to a depth of 50 m into rock, which is conservatively based on the observed air flow in caves. This amounts to an air volume of 220 km^3 that is entering karst rock on a daily basis and is stripped of its ~2 ppmv CH_4 content, which translates to an annual volume of ~0.16 km^3 of microbially oxidized CH_4, or ~11.5×10^7 kg CH_4 per year. This represents ~5% of Vietnam's 2.3×10^9 kg of estimated annual CH_4 emission from rice agriculture and livestock for 2000 (Ministry of Natural Resources and Environment of the Socialist Republic of Vietnam, 2011). An extrapolation to Vietnam's entire karst area of 29,000 km^2 (encompassing karst in northern and central Vietnam with similar geomorphologies; Fig. 1) would account for ~15×10^7 kg CH_4 per year, or oxidation of ~7% of Vietnam's anthropogenic agricultural CH_4 emissions. In spite of the underlying large uncertainty in terms of our assumed daily depth of air exchange (50 m), the porosity of near-surface karst (20%), and the area of limestone exposure (22,000 km^2), we consider our crude estimate to be conservative because our field work did not capture maximum cave ventilation during peak thermal differences between cave air and outside air. The actual amount of annually oxidized CH_4 in Vietnamese karst may well exceed our estimate. Additional experiments on tropical karst of other regions in the world are needed before our Vietnam-based data on CH_4 geochemistry in the vadose zone of karst can be extrapolated globally.

5. Conclusions

The warm, moist, and geomorphologically steep karst in northern Vietnam harbors CH_4-oxidizing bacteria in its air-filled porosity that have access to atmospheric CH_4. The air exchange rate and the microbially mediated oxidation of CH_4 in subterranean karst air are enhanced relative to karst in cooler and less humid climates. The 29,000 km^2 of intensively weathered Vietnamese karst represent a 'natural CH_4 oxidation reactor' that is estimated to remove ~150,000 metric tons of atmospheric CH_4 per year, thus possibly compensating for ~7% of Vietnam's anthropogenic CH_4 emissions from rice agriculture and livestock. Our finding can be qualitatively extrapolated to other tropical karst regions (e.g., Australia, China, Cuba, Indonesia, Jamaica, Malaysia) and thus indicates a significant CH_4 sink in the global atmospheric CH_4 cycle.

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