Microbial contributions to subterranean methane sinks

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ABSTRACT

Understanding the sources and sinks of methane (CH_4) is critical for predicting and managing global biogeochemical cycles. Recent studies have reported that CH_4 concentrations in cave ecosystems are depleted and that these subterranean environments may act as a daily sinks for atmospheric CH_4. It has been hypothesized that this CH_4 depletion may be caused by radiolysis, an abiotic process whereby CH_4 is oxidized via interactions with ionizing radiation derived from radon decay. Alternatively, the depletion of CH_4 concentrations could be due to biological processes, specifically oxidation by methanotrophic bacteria. We theoretically explored the radiolysis hypothesis and conclude that it is a kinetically constrained process that is unlikely to lead to the rapid loss of CH_4 in subterranean environments. We present experimental results to support this claim. We tested the microbial oxidation hypothesis in a set of mesocosm experiments that were conducted in Vietnamese caves. Our results reveal that methanotrophic bacteria associated with cave rocks consume CH_4 at a rate of 1.33 - 2.70 mg $CH_4 \cdot m^{-2} \cdot d^{-1}$. These CH_4 oxidation rates equal or exceed what has been reported in other habitats, including agricultural systems, grasslands, deciduous forests, and Arctic tundra. As such, microbial methanotrophy has the potential to significantly oxidize CH_4 in caves, but also smaller-size open subterranean spaces, such as cracks, fissures, and other pores that are connected to and rapidly exchange with the atmosphere. Future studies are needed to understand how subterranean CH_4 oxidation scales up to affect regional and global CH_4 cycling.

INTRODUCTION

Atmospheric methane (CH_4) is a potent greenhouse gas with rising concentrations that can mainly be attributed to anthropogenic activities (IPCC, 2013; US EPA, 2015). Credible forecasting of global warming by climate models mandates knowledge about the sources and sinks of atmospheric CH_4. One potentially important, but overlooked sink of CH_4 is the oxidation that occurs in subterranean environments. Recent studies have documented that cave ecosystems sometimes have subatmospheric concentrations of CH_4. For example, in a four-year study of St. Michael's Cave in Gibraltar, CH_4 concentrations of cave air were typically 10-fold below atmospheric levels (Mattey *et al.*, 2013). A similar pattern was documented in a set of Spanish caves with some samples having CH_4 concentrations that were below detection limits suggesting near-complete removal of CH_4 from underground air (Fernandez-Cortes *et al.*, 2015).

Two hypotheses have been put forth to explain the pattern of CH_4 depletion in subterranean environments. First, CH_4 is a carbon and energy source that can be used by methanotrophic bacteria. Although methanotrophic bacteria were found in Movile Cave in Romania (Hutchens *et al.*, 2004), microbiological surveys of methane oxidizing bacteria in caves are relatively uncommon. Inferences about methanotrophy in caves have also been made based on evidence from stable isotopes (Peryt *et al.*, 2012). For example, an inverse relationship between CH_4 concentrations and CH_4 carbon stable isotope ratios (i.e., $\delta^{13}C$) in St. Michael's cave was considered a diagnostic signature of methanotrophy (Mattey *et al.*, 2013). A second hypothesis is that CH_4 depletion in subterranean ecosystems is due to radiolysis. This abiotic mechanism of CH_4 oxidation was developed to explain low CH_4 concentrations in a poorly ventilated cave that had a high density of ions, but no recoverable methanotrophic bacteria (Fernandez-Cortes *et al.*, 2015). An inverse correlation between the concentration of CH_4 and

ions in cave air suggests that α-particles from radon decay could contribute to removal of CH_4 from subterranean environments (Fernandez-Cortes *et al.*, 2015).

In this study, we evaluate the relative importance of biotic and abiotic mechanisms that have been put forward to explain low concentrations of CH_4 observed in subterranean environments. First, we develop theoretical expectations in an effort to constrain the rates of radiolytic CH_4 oxidation. Second, we present results from a controlled laboratory experiment aimed at quantifying the effect of ionizing radiation on the rate of CH_4 oxidation. Third, we discuss findings from a set of field mesocosm experiments in Vietnamese caves to quantify the methanotrophic potential of cave microbial communities.

RESULTS AND DISCUSSION

Weak theoretical support for the importance of radiolytic CH_4 oxidation — The following thought experiments reveal that radiolysis is a process that contributes minimally to CH_4 oxidation in subterranean environments on the time scale of days to weeks. We arrive at this conclusion based on the imbalance between the large number of CH_4 molecules and the comparatively small number of radioactive decay events that are typical in caves.

Ionizing radiation in the air of subterranean limestone-based ecosystems is derived predominantly from α-particles that are generated during radon decay (Cigna, 2005; Alvarez-Gallego *et al.*, 2005). These α-particles could lead to the oxidation of CH_4 *via* different mechanisms. For example, radiolysis could result from the direct collision of α-particles with CH_4 molecules. In this case, an α-particle splits a CH_4 molecule, which triggers a subsequent exothermic oxidation reaction of ions and radicals with atmospheric oxygen. However, with a decay rate of ~35,000 ^{222}Rn atoms per second in a cubic meter of air, as measured in a Spanish

92 cave (Fernandez-Cortes *et al.*, 2015), it would take nearly 50 million years to eliminate 2 ppm of CH_4 as a result of direct collision between α-particles and CH_4 molecules.

94 A more likely mechanism occurs when radiogenic energy interacts with water molecules and other major chemical constituents of cave air and thus produces ions and radicals that enter 96 secondary chemical reactions with CH_4. For example, radiolysis of water vapor *via* radon decay could generate hydroxyl radicals (•OH) that act to remove CH_4. However, if every α-decay at 98 35,000 Bq m^{-3} generates $4.3 \cdot 10^5$ ions and radicals (Fernandez-Cortes *et al.*, 2015), it would still require more than 100 years to eliminate 2 ppm of CH_4. In fact, this likely overestimates the 100 potential for radiolytic CH_4 oxidation since the calculations unrealistically assume that all •OH selectively react with CH_4. In sum, our assumptions and calculations suggest that radiolysis is a 102 kinetically constrained process that is unlikely to act as a daily CH_4 sink in subterranean ecosystems (Fernandez-Cortes *et al.*, 2015). More detail on the calculations that were used to 104 arrive at our predictions can be found in the Supplementary Information,

106 **Weak experimental support for the importance of radiolytic CH_4 oxidation —** Results from a laboratory experiment confirm our theoretical predictions by demonstrating that ionizing 108 radiation had a minimal effect on CH_4 oxidation rates. We placed 7.08 g uranium metal powder in a Petri dish on the bottom of a humid polyethylene bag containing 43 L of air with an elevated 110 CH_4 concentration (23.5 ppm). The radioactivity inside the closed bag containing depleted uranium was approximately $2.5 \cdot 10^6$ Bq m^{-3}, which is 70-fold higher than the natural radiation 112 reported in Spanish cave air (Fernandez-Cortes *et al.*, 2015). Yet, in the presence of strong ionizing radiation, CH_4 was lost from the system at the slow rate of 0.197 ± 0.0005 (± standard 114 error) ng $CH_4 \cdot m^{-3} \cdot d^{-1}$, which was indistinguishable from the diffusive loss of CH_4 from

polyethylene control bags lacking uranium (one-sample t-test: $t_6 = -0.97$, $P = 0.37$, Fig.1). More detail concerning experimental procedures can be found in Supplementary Information.

Strong experimental support for the importance of biotic CH_4 oxidation — The results from our field mesocosm experiments suggest that CH_4 depletion can be achieved *via* the biological activity of methanotrophic bacteria that are associated with rocks inside cave ecosystems. In two separate caves with low radon abundances (≤100 Bq m^{-3}) on the island of Cát Bà in Vietnam, we deployed 200-L polyethylene bags filled with cave air containing limestone rocks that were collected from inside the cave. Half of these mesocosms (n = 3) were treated with a 10 % bleach (sodium hypochlorite) solution to inhibit microbial activity ("dead") while the other mesocosms ("live") were treated with an equal volume of water (n = 3). After incubating *in situ* overnight, CH_4 concentrations in the dead mesocosms were indistinguishable from the control mesocosms (no cave rocks) and the cave air (one-sample t-tests, $P > 0.52$, Fig. 2). In contrast, we observed an 87 % ± 0.047 % (mean ± SEM) reduction of CH_4 concentrations in the live mesocosms.

From our experimental data, we estimate that the rate of CH_4 oxidation associated with cave rocks was between 1.33 and 2.70 mg $CH_4 \cdot m^{-2} \cdot d^{-1}$. To the best of our knowledge, these are the first direct measurements of biological CH_4 oxidation in a cave ecosystem. The magnitude of these rates equals or exceeds the rates of CH_4 oxidation that have been reported in soils from agricultural systems, grasslands, mature forests, and Arctic tundra (Whalen & Reeburgh, 1990; Suwanwaree & Robertson, 2005; von Fischer *et al*., 2009). This comparison is noteworthy because caves maintain relatively constant temperatures thoughout the year, while soils in mid-to-high latitudes often experience lower temperatures during the winter season, which results in reduces rates of CH_4 oxidation (e.g., Groffman *et al*., 2006). As such, when integrated over

138 annual time scales, subterranean enviornments like caves may act as a larger sink for atmospheric CH_4 than has been previously recognized.

140 Our experiments revealed that methanotrophic bacteria were abundant in the biofilms that were associated with Vietnamese cave rocks. We conducted quantitative PCR assays on DNA 142 extracted from rocks that were incubated in the live mesocosms using primers that targeted the particulate methane monoxygenase (*pmo*A) gene, which is responsible for bacterial CH_4 144 oxidation (see Supplementary Information for more detail). From this, we recovered $1.0 \cdot 10^4$ to $1.5 \cdot 10^4$ *pmo*A gene copies per gram of rock biofilm. When standardized by 16S rRNA gene 146 copy number, we estimate that the relative abundance of methanotrophs in the cave biofilms ranged from 0.16 to 1.48 % of the microbial community.

148 Despite recent global-scale efforts to survey the diversity of microbial communities from a wide range of habitats, reports of methane oxidizing bacteria from cave ecosystems are scarce. 150 For example, using cultivation-independent approaches, no sequences closely matching known methanotrophs were recovered from the Frasassi cave complex in central Italy (Macalady *et al.*, 152 2006). Methanotrophs were recovered from some, but not all Spanish caves (Fernandez-Cortes *et al.*, 2015). In limestone caves of Kartchner Caverns, Arizona (USA), a single sequence was 154 recovered that was closely related to *Methylocella*, which is a facultative methanotroph (Ortiz *et al.*, 2013). Similarly, only one sequence from the walls of a karstic cave in Slovenia was closely 156 related to *Methylococcus*, which is an obligate methanotroph (Pašič *et al.*, 2010). In contrast, the presence and activity of methanotrophs was documented in water and mat samples collected 158 from Movile Cave using stable isotope probing (SIP). In this study, researchers tracked ^{13}C-labeled CH_4 into the DNA of bacteria that were closely related to known methanotrophs such as 160 *Methylomonas*, *Methylococcus*, and *Methylocystis*/*Methylosinus* (Hutchens *et al.*, 2004). Given

their potential role in consuming subterranean CH_4, more studies are needed to characterize the diversity and activity of methanotrophs in a wider range of cave ecosystems.

In the methane-depleted Castañar Cave in Spain, the importance of methanotrophy was ruled out based on the assumption that bacteria would not be able to meet their metabolic demands for maintenance and growth (Fernandez-Cortes *et al.*, 2015). However, this argument overlooks two important ecophysiological features of microorganisms in natural ecosystems. First, growing evidence suggests that many microorganisms can tolerate extreme energy limitation on timescales ranging from centuries to millennia (Hoehler & Jørgensen, 2013) owing to life-history strategies such as dormancy (Lennon & Jones, 2011). Second, microorganisms in nature are commonly challenged with "feast or famine" conditions. For example, the supply of CH_4 to cave habitats varies through time depending on the source of CH_4, seasonality, ventilation, microclimatic conditions, and geography. Methanotrophic bacteria in caves are likely adapted to such fluctuations in CH_4 concentrations, which are not captured with synoptic sampling.

Conclusion — Although ionizing radiation can accumulate in poorly vented, deep recesses of some caves, this is neither necessary nor sufficient to explain the pattern of CH_4 depletion in subterranean ecosystems. Both theoretical and experimental lines of evidence suggest it is unlikely that radiolytically induced CH_4 oxidation serves as a significant mechanism for rapid depletion of CH_4 in cave air. Rather, our results support the hypothesis that bacterial methanotrophy alone has the potential to significantly oxidize CH_4 in caves, but also smaller-size open subterranean spaces, such as cracks, fissures, and other pores that are connected to the atmosphere.

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FIGURE CAPTIONS

Fig. 1. Rates of methane (CH_4) oxidation were not significantly affected by ionizing radiation. We conducted a laboratory experiment where we tracked the concentration of CH_4 in a polyethylene bag containing air and ionizing radiation from a source of uranium metal powder (black line, n = 1) to the concentration of CH_4 in control bags without an added source of ionizing radiation (grey lines, n = 7). We attribute the slow loss of CH_4 in all trials to diffusion through polyethylene bag.

Fig. 2. Field mesocosm experiments in two Vietnamese caves support the biological methane (CH_4) oxidation hypothesis. Control mesocosms contained no cave rocks and provided an estimate for the diffusive loss of CH_4; "dead" mesocosms contained cave rocks that were treated with a 10 % bleach solution; "live" mesocosms contained cave rocks and a volume of water (150 mL) equivalent to the volume of bleach used in the “dead” treatment. The dashed horizontal lines correspond to the CH_4 concentrations in the Hoa Cương and Minh Châu caves on Cát Bà island, northern Vietnam.

Fig. 1.

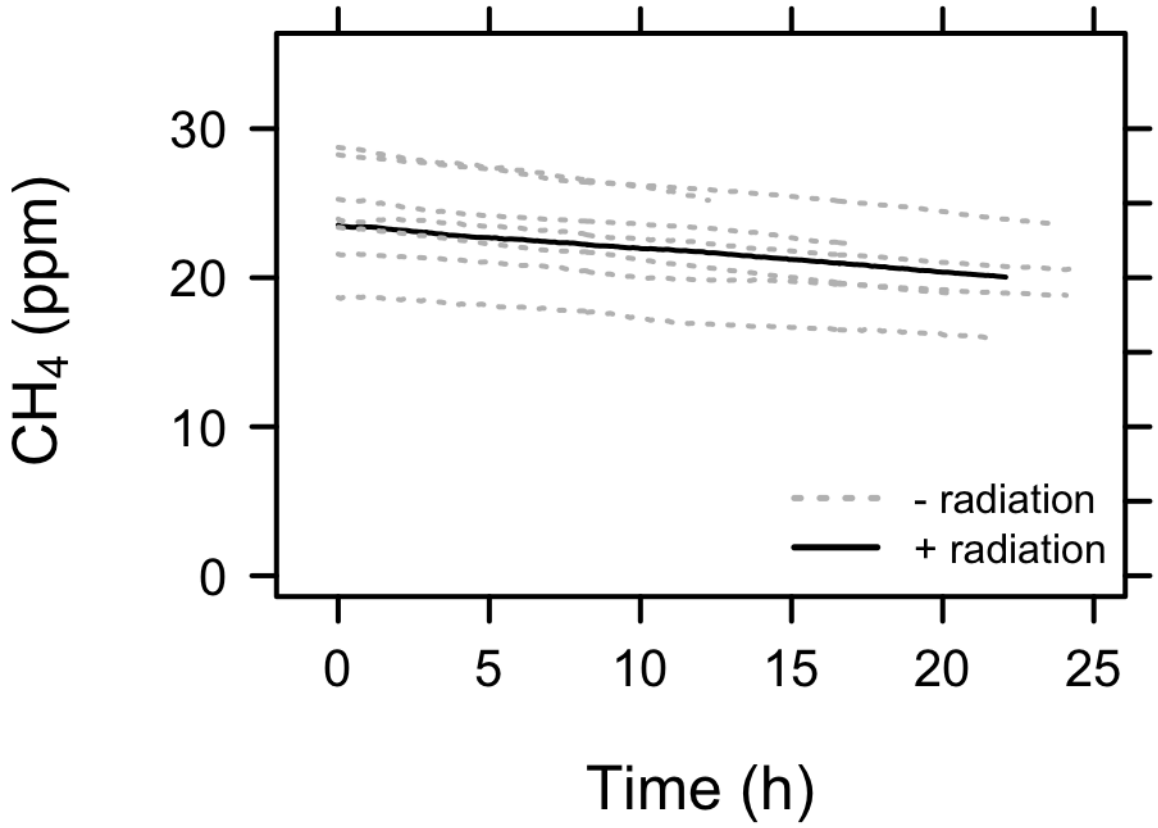

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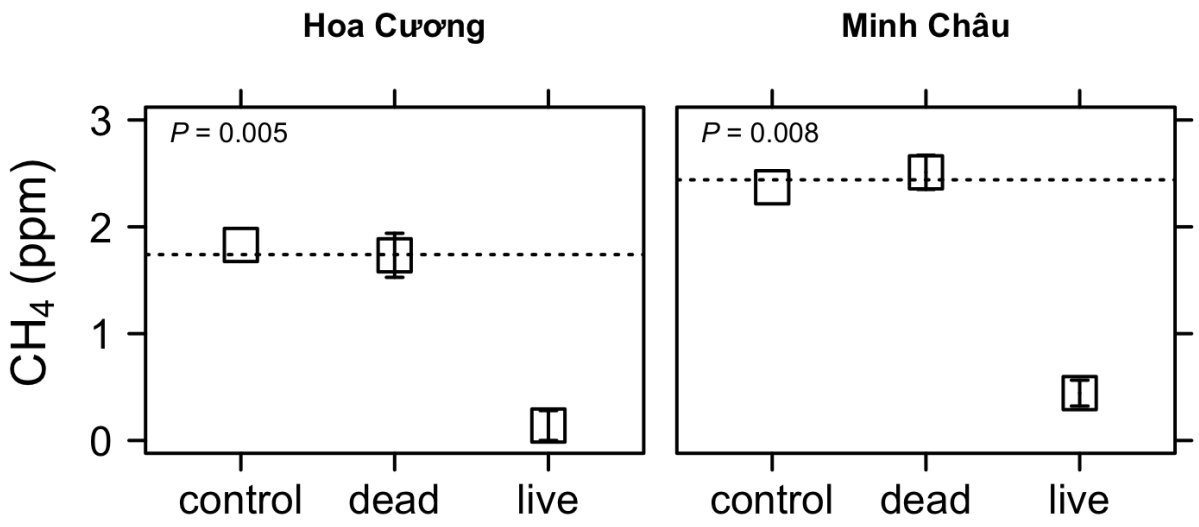
Fig. 2.



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