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Letter

Isotopic Labeling Reveals Microbial Methane Oxidation Coupled to Fe(III) Mineral Reduction in Sediments from an As-Contaminated Aquifer

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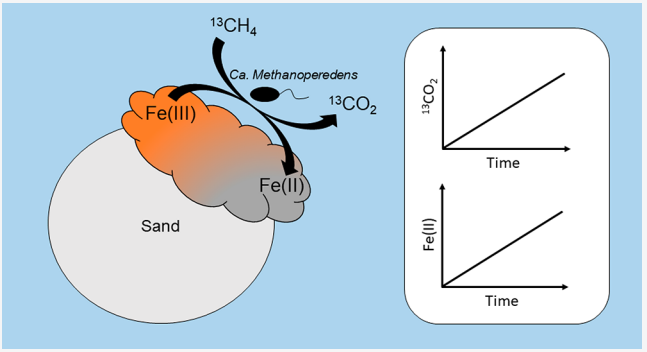
ABSTRACT: Although arsenic (As) groundwater contamination in South and Southeast Asia is a threat to human health, mechanisms of its release from sediment to groundwater are still not fully understood. In many aquifers, Fe(III) minerals are often the main hosting phases for As and their stability is crucial for As mobility. Recently, a new mechanism for As mobilization into groundwater was proposed with methane (CH_4) serving as an electron donor for microbially mediated reductive dissolution of As-bearing Fe(III) minerals. To provide unequivocal evidence for the occurrence of Fe(III)-coupled methanotrophy, we incubated sediments from an As-contaminated aquifer in Hanoi (Vietnam) anoxically with isotopically labeled $^{13}CH_4$. Up to 35% of the available Fe(III) was reduced within 232 days with simultaneous production of $^{13}CO_2$ demonstrating anaerobic oxidation of $^{13}CH_4$ with Fe(III) as the electron acceptor. The microbial community at the end of the incubation was dominated by archaea affiliating with *Candidatus* Methanoperedens, implying its involvement in Fe(III)-dependent CH_4 oxidation. These results suggest that methanotrophs can contribute to dissolution of As-bearing Fe(III) minerals, which eventually leads to As-release into groundwater.



INTRODUCTION

Arsenic (As) groundwater contamination is a worldwide threat to human health. It is estimated that up to 220 million people live in areas where As-concentration in groundwater exceeds the World Health Organization (WHO) drinking water threshold value of 10 μg/L, of which 85−90% live in South Asia.[1] In this area, groundwater recharge in shallow, Holocene aquifers is influenced by rivers that drain the Himalayas and carry eroded sediment coated with As-rich Fe(III) (oxyhydr)-oxide minerals.[2] Microbial reductive dissolution of these As-bearing Fe(III) minerals coupled to oxidation of organic carbon (OC) in anoxic aquifers is considered to be one of the key mechanisms of As-release from the solid to aqueous phase.[3−6] A wide spectrum of organic compounds was examined as electron donor, including easily bioavailable acetate,[7] glucose,[8] and environmentally relevant sources such as *in situ* organic matter.[9] However, until recently, the potential of the simplest hydrocarbon—methane (CH_4)—to serve as electron donor and to drive the release of As from the sediments was still unexplored.

Both bacteria and archaea have been described to oxidize CH_4, but for a long time it was believed that the former carry out the reaction aerobically while the latter do it anaerobically.[10] The subunit A of the particulate methane monooxygenase (*pmoA*) gene is involved in the initial conversion of CH_4 into methanol (CH_3OH) in the presence of oxygen and it is present in most aerobic methanotrophic bacteria.[11] Therefore, it has been used as a biomarker to track bacterial methanotrophic communities and their activity.[12] Recently, abundant and active aerobic methanotrophs have been found in anoxic environments, suggesting that these microorganisms may use other electron acceptors than oxygen.[13] Anaerobic methanotrophic archaea use a different metabolic pathway for CH_4 oxidation, called "reverse methanogenesis". As its name indicates, it operates in the reverse direction than earlier discovered methanogenesis.[14,15] This applies also to the *mcrA* (methyl coenzyme M reductase A) gene which in the "forward" direction catalyzes the terminal step of methanogenesis, while in the "reverse" direction it is an essential first step of anaerobic CH_4 oxidation.[16] So far, three concepts have

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been proposed to explain the reversibility of *mcrA*: (i) high CH_4 concentration; (ii) specific modifications within the methyl coenzyme M reductase A (*mcrA*) structure; (iii) high abundance of *mcrA* gene in a cell.[16] In contrast to aerobic methanotrophic bacteria, anaerobic archaea are known to use a wide range of electron acceptors including NO_3^-,[17] SO_4,[2−19] As(V),[20] Mn(IV) and Fe(III).[21]

Recently, using the same type of sediment from the same study site, we have shown that As mobilization via anaerobic CH_4 oxidation coupled to As-bearing Fe(III) minerals reduction can lead to As mobilization.[22] However, the concentrations of CH_4 and CO_2 were not monitored and CH_4 oxidation was only postulated based on microbial community analysis that showed high relative abundance of methanotrophs and increased gene abundance related to CH_4 oxidation (i.e., *pmoA* and *mcrA*). The aim of the present study was therefore to provide unequivocal evidence whether the microbial community present in an As-contaminated aquifer is capable of anaerobically oxidizing CH_4 using Fe(III) as electron acceptor. For this purpose, a Fe(III)-rich sediment from an As-contaminated aquifer in NE Vietnam was anoxically incubated with ^{13}C-labeled CH_4. We monitored Fe(II) as well as $^{13}CO_2$ evolution over time. Furthermore, we analyzed *pmoA* and *mcrA* gene copy numbers at the end of the experiment and performed 16S rRNA gene amplicon sequencing.

MATERIAL AND METHODS

Field Site and Sample Collection. The sampling site is located about 15 km SE from Hanoi, Vietnam in Van Phuc village, on a meander bend of the Red River (20°55′18.7″N, 105°53′37.9″E). A Holocene aquifer in the vicinity of the river is characterized by strongly reducing conditions (which is manifested by the gray color of sediment) and high concentrations of As in groundwater (up to 60 times higher than WHO guideline[23]). Urban treatment facilities extract water with low As concentration (below WHO guideline) from the neighboring aquifer, which consists of older, Pleistocene Fe(III)-rich orange-brownish sediment.[24] The interface between the two aquifers is characterized by a changing redox condition (from highly to moderately reducing) and thus is referred to as a redox transition zone (RTZ).[25] Previous studies detected high CH_4 (11−38 mgC/L) and rather low DOC (1.1−4.4 mgC/L) concentrations in As-contaminated groundwater at the RTZ.[26] In October 2018, a drilling campaign took place in this zone. Sediment cores were collected (10 cm diameter; ca. 3 m long pieces) up to 46 m below the ground surface using the rotary drilling method. The sediment was stored anoxically at 4 °C in the dark until further use (15 months). For our microcosm experiment, the orange sediment from 30 m depth was taken (Figure S1) because preliminary data showed that this sediment had high As and Fe contents and therefore could be a source for their release to the groundwater. Moreover, our previous study showed a higher abundance of the putative Fe(III)-reducing methanotroph *Candidatus* Methanoperedens and confirmed the prevalence of easily bioavailable Fe(III) minerals such as $Fe(OH)_3$ at this depth[27] making these sediments suitable for Fe(III)-reducing CH_4 oxidation studies.

Experiment Setup. Microcosms were set up by mixing 30 g (wet weight) of sediment with 100 mL of sterile Milli-Q water in 250 mL autoclaved serum glass bottles. The Milli-Q water was used to avoid introduction of bicarbonate buffer that would influence our gas measurements. The following microcosm treatments were prepared in triplicate: (1) biotic, with addition of ^{13}C labeled CH_4 (Sigma-Aldrich, 99 atom % ^{13}C) to the headspace of each bottle (100% $^{13}CH_4$); (2) CH_4-free biotic microcosms, without any further additives; (3) abiotic controls, with the addition of CH_4 and sodium azide (160 mM) inhibiting microbial activity. One setup of the abiotic controls contained ^{13}C-labeled CH_4 (100% $^{13}CH_4$) while the other two controls contained CH_4 with natural isotope abundance (99% $^{12}CH_4$, 1% $^{13}CH_4$). All microcosms were prepared in a glovebox (99.999% N_2) and sealed to maintain anoxic conditions. The headspace was exchanged with $^{13}CH_4$ or CH_4, with the exception of the CH_4-free microcosms where N_2 remained as the main gas phase. The pressures inside the bottles were measured with a portable monometer (Supporting Information Table S.1). During the experiment, microcosms were incubated at 26 °C (corresponding to aquifer temperature) in the dark and without shaking. Samplings were performed every one to 2 weeks.

Geochemical Analysis. *Gas Phase.* For the $^{13}CO_2$ sampling, 2 mL of the microcosm's gas phase was withdrawn with a glass Hamilton syringe and sterile needle and injected to a 20 mL headspace vial in an anoxic glovebox (99.999% N_2, purity level 5.0). The headspace vials were sealed with butyl-rubber stopper and aluminum crimp cap before each sampling and contained a glovebox atmosphere with 0.1 bar overpressure (to prevent underpressure formation during gas withdrawal for analysis). Fresh sets of blanks and standards were prepared before each sampling. Blanks contained 100% of N_2 (glovebox atmosphere), while standards were made by diluting pure CO_2 in N_2. Concentrations of $^{13}CO_2$ were measured on the same day as sample collection using a TraceGC 2000 (Thermo Finnigan, Mailand, Italy) gas chromatograph coupled to a mass spectrometer (Trace DSQ II, Thermo Finnigan, Austin TX, USA). Injection of 2.5 mL at 100 °C was performed with a split ratio of 1:5. Gases were separated on a micropacked ShinCarbon ST column (100/120 mesh 2 m, 1 mm ID, Restek GmbH, Germany) with helium as the carrier gas, at a flow rate of 2 mL min^{-1} and the following temperature program: 40 °C hold for 1 min, ramped by 10 °C min^{-1} to 1750 °C and hold 1 min. The sampling error for $^{13}CO_2$ was determined as 5% and a detection limit of 0.5 ppm. To calculate total moles of $^{13}CO_2$ in an incubation bottle, a correction for gas−water partitioning and cumulative sample removal were applied (Supporting Information).

Liquid and Solid Phases. Samples were collected with sterile syringes and needles (inner diameter of needle = 1.37 mm) inside a glovebox. 1.5 mL of slurry (a mixture of water and sediment) was taken from each bottle and centrifuged at 14 000 rpm for 5 min. Then 100 μL of the supernatant was mixed with 100 μL of 1 M HCl (to prevent Fe(II) oxidation) and stored for aqueous Fe^{2+} quantification with the ferrozine assay.[28] The sediment obtained after centrifugation was digested for 1 h with 1 mL of 6 M HCl. Afterward, the sample was centrifuged (5 min, 14 000 rpm), and 100 μL of the supernatant was diluted in 1 M HCl and saved for Fe(II) and Fe(tot) analysis with the ferrozine assay.[28] Fe(tot) was quantified using hydroxylamine hydrochloride as a reductant. Samples with sodium azide were acidified under the fume hood in order to prevent the release of toxic hydrogen azide and handled with extra precautions. To avoid the changes in the internal bottles' pressure, after each sampling the withdrawn

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volume (including gas, aqueous and solid phase) was replaced with an equal volume of N_2.

Molecular Analyses. Total genomic DNA was extracted from all samples (original sediment and sediment after 232 days of incubation) using an up-scaled version of the phenol–chloroform protocol following Lueders et al.[29] at the end of the experiment.

16S rRNA Gene Amplicon Sequence Analysis. Library preparation steps and sequencing were performed by Microsynth AG (Switzerland) as detailed in the Supporting Information. Sequence analysis was performed with nf-core/ampliseq v1.2.0[30,31] and is described in detail in the Supporting Information. Raw sequencing data can be found at the NCBI Sequence Read Archive; accession number PRJNA719005).

Quantitative PCR. Bacteria and archaea were estimated by quantifying their 16S rRNA gene copy numbers via qPCR using a C1000 Touch thermal cycler (CFX96 real time system). To estimate the abundance of methanotrophic bacteria and archaea, particulate methane monooxygenase (*pmoA*) and methyl coenzyme M reductase (*mcrA*) genes were quantified, respectively. Reaction mixes were prepared following the SybrGreen Supermix kit (Bio-Rad Laboratories GmbH, Munich, Germany). Primers, PCR reaction details, and bacterial standard templates are detailed in the Supporting Information (Table S.2). Each sample was measured in technical triplicates and the specificity of the amplification was assessed by melting curve analysis. Concentration of the calibration standard DNA template was measured with ND-2000 UV–vis spectrophotometer (NanoDrop Co., USA). Data analysis was done using the Bio-Rad CFX Maestro 1.1 software version 4.1 (Bio-Rad, 2017).

RESULTS AND DISCUSSION

Fe(III) Reduction Coupled to Anaerobic CH_4 Oxidation. The reduction of Fe(III) to Fe(II) in the $^{13}CH_4$-amended biotic microcosm was manifested by the color change of sediments from orange to gray (Figure 1a). During the experiment, aqueous Fe^{2+} was absent in all microcosms. It must be kept in mind that using Milli-Q water changed the ionic strength of the solution. Moreover, the sediments used here are characterized by a strong sorption capacity. In consequence, neither Fe^{2+} nor As were released into the water but were retained in the solid phase (please see SI file). Lack of Fe^{2+} in the solution could be also due to analytical limitations (detection limit of 25 μM) or precipitation of siderite. However, continuous reduction of Fe(III) and formation of solid-phase Fe(II) was observed (Figure 1b). During 232 days of incubation, the amount of Fe(II) in the sediment increased from 0.0044 ± 0.0001 to 0.22 ± 0.045 mmol/microcosm (average from 3 bottles), which corresponds to 3% and 35% of the total solid-phase Fe, respectively. Concurrently, $^{13}CO_2$ was being produced steadily in the $^{13}CH_4$-amended biotic microcosms, reaching 2.0 ± 0.2 mmol/microcosm (considering gas–water partitioning and cumulative sample removal; Figure 1c). Please note that we provide the data as absolute amounts (mmol/microcosm) rather than concentration (mol/L) to account for the fact that $^{13}CO_2$ is partitioned between liquid and gas phases. For easier comparison, the same unit is used for Fe(II).

The concomitant increase of Fe(II) and $^{13}CO_2$ indicates that $^{13}CH_4$ oxidation was coupled to Fe(III) reduction. The ~0.1:1 stoichiometry of Fe(II) and $^{13}CO_2$ production (ΔFe(II)/$\Delta^{13}CO_2$ = 0.11) did not fit to the theoretical stoichiometric

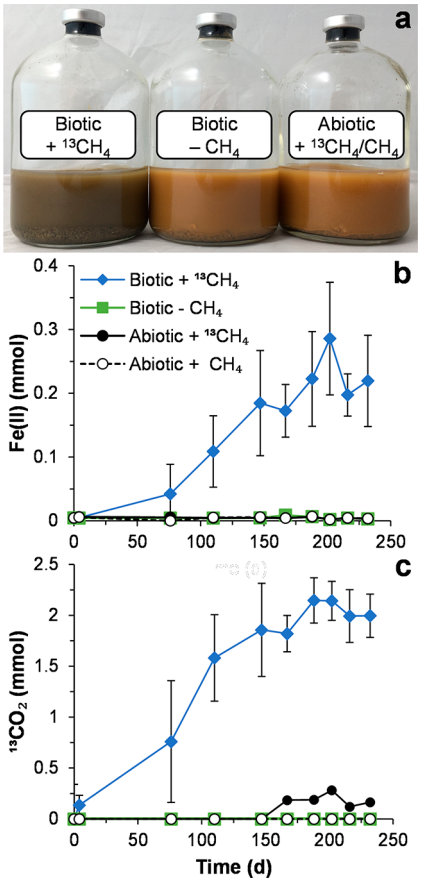

Figure 1. Changes in solid-phase Fe(II) and $^{13}CO_2$. (a) Color of sediment at the end of the experiment (day 232). Only the "Biotic + $^{13}CH_4$" setup changed color from orange to gray indicating a change of Fe redox state from Fe(III) to Fe(II). Control setups remained orange. (b) Absolute amount of solid-phase Fe(II) and (c) $^{13}CO_2$ in the microcosm over 232 days of incubation of As-bearing sediments supplied with $^{13}CH_4$ compared to biotic and abiotic control setups. To account for the fact that $^{13}CO_2$ is partitioned between liquid and gas phases, we use absolute amount (mmol/microcosm) for $^{13}CO_2$ and Fe(II). Please note that control symbols mostly cover each other (with concentrations at a value of 0). Error bars represent standard deviation from three replicates.

ratio of 8:1 previously observed for Fe(III)-dependent methane oxidation.[32] However, here instead of a pure microbial culture and pure Fe(III) mineral, a sediment containing a mixed bacterial-archaeal community and mixed mineral phases with other geochemical constituents was used and therefore other Fe-and C-cycling processes likely took place influencing the final ratio of $^{13}CO_2$ and Fe(II). For example, part of $^{13}CH_4$ may be oxidized using other electron acceptors such as Mn(IV), As(V),[20,33] or trace concentration of O_2, overall increasing the final amount of $^{13}CO_2$ without influencing Fe cycling. It should also be considered that some portion of the produced $^{13}CO_2$ could be present in solution in the form of (bi)carbonate, precipitated as siderite or be assimilated as microbial biomass leading to further deviation from theoretical mass balance.

While there was no $^{13}CO_2$ production in the biotic control, a minor production of $^{13}CO_2$ in the $^{13}CH_4$-amended abiotic control was observed (0.18 mmol/microcosm Figure 1c); however, it was significantly lower than $^{13}CO_2$ production in the $^{13}CH_4$-amended biotic microcosm. Since it took place after

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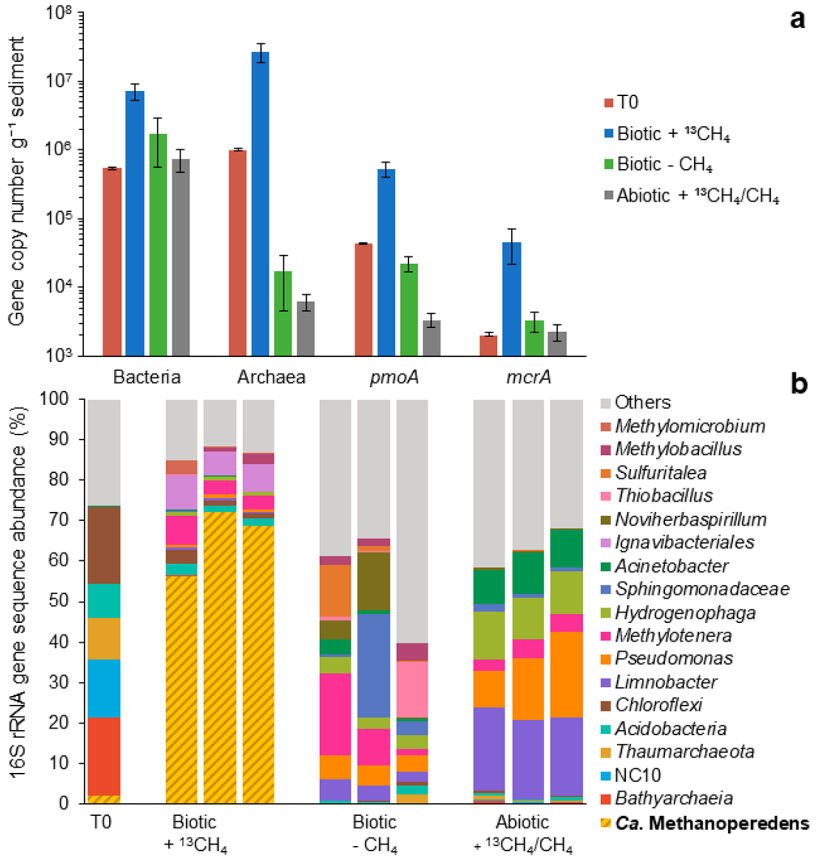

Figure 2. Gene abundance and microbial community composition in microcosms after 232 days of incubation. (a) Quantitative PCR of bacterial and archaeal 16S rRNA genes, as well as *pmoA* and *mcrA* genes in 1 g of wet sediment. (b) Relative abundance of microbial taxa based on 16S rRNA gene amplicon sequencing in original sediments (T0) and in three different treatments analyzed in biological triplicate.

150 days of incubation, this could be caused by decreasing inhibition effectiveness of sodium azide over time, which would allow for low microbial activities at the end of the incubation ($^{13}CH_4$ oxidation or degradation of organic matter present in sediment). However, the content of Fe(II) remained stable among all control microcosms, indicating that Fe redox changes took place only in the presence of both CH_4 and an active microbial community. Altogether, remarkable Fe(II) and $^{13}CO_2$ production took place only in active $^{13}CH_4$-amended microcosm, which confirms that the microbial community from the sediment is capable of reducing Fe(III) using CH_4 as an electron donor.

Molecular Evidence for Microbially Mediated Methane Oxidation. At the end of the experiment, the microbial community in $^{13}CH_4$-amended biotic microcosms was highly enriched in archaea compared to that in other setups and compared to that in the original sediments (2.7×10^7 archaeal 16S rRNA genes g^{-1} wet sediment; Figure 2a). Noteworthy, only low numbers of archaea were detected in both controls (ca. 7.1×10^3 archaeal 16S rRNA genes g^{-1} wet sediment), indicating that CH_4 was the main factor stimulating growth of these microorganisms. Archaea affiliating to *Candidatus* Methanoperedens, initially representing only 2% of relative 16S rRNA gene abundance in the original sediment, dominated all three CH_4-amended microbially active microcosms, reaching 55−75% of the microbial community at the end of the experiment (Figure 2b). *Ca.* Methanoperedens was previously identified in the same sediment as studied here[26] and was shown in other studies to mediate Fe(III)-dependent CH_4 oxidation via the reverse methanogenesis pathway.[32,34] The *mcrA* gene abundance was highest in the CH_4-amended biotic microcosm as well (4.5×10^4 copies g^{-1} wet sediment; Figure 2a), implying that anaerobic CH_4 oxidation was likely taking place. Although the *pmoA* gene was also abundant in CH_4-amended biotic microcosms (5.3×10^5 copies g^{-1} wet sediment; Figure 2a), the dominance of archaeal 16S rRNA genes and *Ca.* Methanoperedens harboring *mcrA* genes indicates that this archaeon was a key player in Fe(III)-dependent CH_4 oxidation. The increase of *pmoA* in our experiment, which was conducted under anoxic conditions, implies that either microorganism harboring this gene can use other electron acceptors than O_2 or that trace concentrations of O_2 were introduced at the sampling time, which stimulated the growth of aerobic methanotrophs. Nevertheless, the high abundance of archaea and the enrichment of *Ca.* Methanoperedens provide strong evidence that Fe(III) was reduced coupled with anaerobic methanotrophy.

Environmental Significance. Fe(III) (oxyhydr)oxides are the main As-bearing mineral phases in many CH_4-containing aquifers across SE Asia, including the Van Phuc aquifer.[35] Our study shows that Fe(III)-dependent CH_4 oxidation has the potential to be an important pathway for As mobilization in the studied area. This finding is particularly relevant in the context of the stability of the redox transition zone in the Van Phuc aquifer. The establishment of methanogenic conditions within the redox transition zone could cause As release into groundwater and contamination of yet As-free Pleistocene aquifer, which is used as a main source of potable water.

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Moreover, high CH_4 concentrations within As-groundwater hotspots have been reported at many locations in SE Asia such as the Bengal, Mekong and Red River deltas,[25,36−38] highlighting a potentially important role of CH_4 as a driver of As mobilization, leading to groundwater contamination on a regional and possibly global scale.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.1c00553.

Sampling and experimental details, correction for gas−water partitioning and cumulative sampling, sediment sorption capacity calculation (PDF)

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Notes

The authors declare no competing financial interest.

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