Methanogens rapidly transition from methane production to iron reduction

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ABSTRACT

Methanogenesis, the microbial methane (CH4) production, is traditionally thought to anchor the mineralization of organic matter as the ultimate respiratory process in deep sediments, despite the presence of oxidized mineral phases, such as iron oxides. This process is carried out by archaea that have also been shown to be capable of reducing iron in high levels of electron donors such as hydrogen. The current pure culture study demonstrates that methanogenic archaea (Methanosarcina barkeri) rapidly switch from methanogenesis to iron-oxide reduction close to natural conditions, with nitrogen atmosphere, even when faced with substrate limitations. Intensive, biotic iron reduction was observed following the addition of poorly crystalline ferrihydrite and complex organic matter and was accompanied by inhibition of methane production. The reaction rate of this process was of the first order and was dependent only on the initial iron concentrations. Ferrous iron production did not accelerate significantly with the addition of 9,10-anthraquinone-2,6-disulfonate (AQDS) but increased by 11–28% with the addition of phenazine-1-carboxylate (PCA), suggesting the possible role of methanophenazines in the electron transport. The coupling between ferrous iron and methane production has important global implications. The rapid transition from methanogenesis to reduction of iron oxides close to the natural conditions in sediments may help to explain the globally-distributed phenomena of increasing ferrous concentrations below the traditional iron reduction zone in the deep ‘methanogenic’ sediment horizon, with implications for metabolic networking in these subsurface ecosystems and in past geological settings.

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INTRODUCTION

During the anaerobic oxidation of organic matter in aquatic sediments, microbial dissimilatory respiration is performed by the reduction of electron acceptors along a cascade of decreasing free energy yield (Froelich et al., 1979). The greatest energy yield among common processes is associated with nitrate reduction (denitrification) followed by bacterial manganese and iron-oxide reduction and then sulfate reduction. Below the depth of sulfate depletion, microbial methane (CH4) production (i.e., methanogenesis) traditionally serves as the ultimate biological process anchoring carbon remineralization in deep sediments (Froelich et al., 1979).

Bacterial sulfate reduction is responsible for the majority of organic matter oxidation in marine sediments (Kasten & Jorgensen, 2000). In most freshwater environments, where sulfate concentrations are low, dissimilatory iron-oxide reduction and methanogenesis are the dominant processes involved in the anaerobic degradation of organic carbon (e.g., Roden & Wetzel, 1996). Denitrification has also been recognized to be a dominant electron acceptor in some water bodies and sediments. It has been shown that these dissimilatory anaerobic processes can co-exist in sediments (e.g., Sivan et al., 2014) and in the water column (Crowe et al., 2011), indicating that the traditional concept of strict dissimilatory respiration order is oversimplified, because it assumes no availability limitation of the electron acceptors (as Fe(III) or Mn(IV) oxides) or the electron donor. However, the coupling between the various processes, particularly the iron and methane cycles, remains enigmatic, even with the growing literature on the subject (see below). Exploring how these processes may be linked may have important implications for our understanding of the subsurface carbon cycle. Dissimilatory iron-oxide reduction plays an important role in the reductive
dissolution of iron minerals in the natural environment. The ability of bacteria to use iron oxides as terminal electron acceptors for respiration was shown in the Gram-negative bacteria (Lovley & Phillips, 1988; Myers & Nelson, 1988) in Gram-positive bacteria and archaea (Lovley, 2006; Weber et al., 2006). Iron reduction presents a challenge to micro-organisms because iron oxides are poorly soluble in most aqueous environments, where the pH is circum neutral, and microbial cells are impermeable to iron oxides (Shi et al., 2007). To overcome this physical barrier, several electron transfer strategies for the extracellular reduction of iron minerals have evolved: (i) a transfer of electrons from the inner membrane to the surface of the cell and to the mineral by electron carriers such as c-type cytochromes [e.g., Geobacter (Mehta et al., 2005)]; (ii) a transfer of electrons through a cellular appendage, such as an electrically conductive pilus or nanowire, that forms a bridge to the mineral (Gralnick & Newman, 2007; Shi et al., 2007; Gorby et al., 2009); in Geobacter it was shown that aromatic amino acids are required for pili conductivity (Vargas et al., 2013), and in Shewanella oneidensis strain MR-1 the nanowires were found to be extensions of the outer membrane and periplasm that include the cytochromes responsible for the electron transport (Pirbadian et al., 2014); (iii) production of complexing ligands (siderophores) (Lippard & Berg, 1994) by the cells that are able to solubilize the iron oxides that are then reduced on the outside or inside of the cells; and (iv) using electron shuttles such as cysteine (Doong & Schink, 2002) and humic acids (Lovley et al., 1996) or endogenously produced redox-active metabolites (Hernandez et al., 2004). The electron shuttles are cyclically reduced by the cell and oxidized by the iron oxides. Strategy 4 may require that the bacteria themselves produce small organic compounds necessary to permit extracellular electron shuttling between the cell and the mineral [like menaquinones (Newman & Kolter, 2000)]. Strategies 3 and 4 may be advantageous as the iron substrate remains at a distance from the cell.

The reactivity of iron minerals used by bacteria as electron acceptors has been explored, and it seems that variations in mineral properties result in a continuum in reactivity (e.g., Munch & Ottow, 1983; Lovley & Phillips, 1986). Munch & Ottow (1983) showed that amorphous ferric iron [\(\text{Fe(III)}(\text{OH}_3)\)] is reduced preferentially over crystalline phases of \(\text{Fe(III)}\). Among the crystalline phases, the reactivity decreases from lepidocrocite to hematite to goethite. Lovley & Phillips (1986) showed that while amorphous iron oxhydroxides are microbially readily reducible, magnetite, and mixed valence state \(\text{Fe(III)}-\text{Fe(II)}\) compounds as well as most of the other oxalate-extractable ferric iron are not available for microbial reduction.

Methanogenesis occurs widely in anoxic natural environments, such as sediments and wetlands and has an important role in global carbon cycling (Whiticar et al., 1986). Acetate and \(\text{H}_2/\text{CO}_2\) are the most important immediate pre-cursors for methane production. In marine sediments, the dominant pathway for methanogenesis is \(\text{CO}_2\)-reduction by hydrogen (hydrogenotrophic methanogenesis), while methanogenesis in freshwater environments proceeds mainly through acetate fermentation (acetoclastic methanogenesis) (Whiticar et al., 1986). When the produced methane comes into contact with an available electron acceptor, it can be consumed by microbial oxidation (methanotrophy), the main process by which methane is prevented from escaping into the atmosphere. In the terrestrial environment, bacteria are mainly responsible for oxidizing methane to \(\text{CO}_2\) using \(\text{O}_2\) as the electron acceptor (Chistoserdova et al., 2005). In marine sediments archaea consume the majority of upward diffusing methane anaerobically through oxidation of methane (AOM) coupled to sulfate reduction (Valentine, 2002). AOM has also been shown to occur via denitrification (Raghoebarsing et al., 2006; Ettwig et al., 2009, 2010; Haroon et al., 2013; Noröi & Thamdrup, 2014; Shen et al., 2015).

Iron-driven AOM was evident in seep sediments (Beal et al., 2009; Sivan et al., 2014), brackish coastal sediments (Egger et al., 2015) and lake water column (Crowe et al., 2011), coupling iron and the methane cycles. Field and laboratory evidence has suggested AOM via the reduction of mineral-bound iron also in the deep methanogenic zone in sediments (Sivan et al., 2007, 2011; Riedinger et al., 2014). This was shown using sedimentary profiles, modeling and incubation experiments. Until these studies only few microbial respiratory pathways other than methanogenesis had been demonstrated in deep sediments below the sulfate reduction zone, although evidence suggests trace biological production of ethane and propane occurs by as-yet unknown processes (Hinrichs et al., 2006). Despite the presence of oxidized mineral phases, such as iron oxides, which also could be important electron acceptors for microbiota for the continued oxidation of organic carbon (e.g., Roden & Wetzel, 1996; Kotka et al., 2002; Straub & Schink, 2003), deeper sediments were presumed to be largely microbially inactive. The mentioned physical barriers may limit the rate and extent of iron availability (e.g., Lovley & Phillips, 1986; Canfield, 1989; Postma, 1993; Shi et al., 2007). Thus, this deep iron-driven AOM would necessitate a direct coupling between the iron and methane cycles in the subsurface that overcomes the limitations of the iron-oxide compounds. Sivan et al. (2011) suggested that in certain conditions iron oxides may be more available for reduction by methane oxidation than by other types of organic compounds. This is either by the presence of methane that may be more available or by the methanogens and their electron shuttles.

There also seems to be an enigmatic link between oxidized iron and methanogenesis. Previous observations demonstrated the inhibition of methanogenesis under
iron-reducing conditions due to competition between methanogens and iron-reducing bacteria for the common substrates acetate and hydrogen (e.g., Lovley & Phillips, 1986; Roden & Wetzel, 1996; Conrad, 1999; Roden, 2003) and direct iron-utilization by methanogens (Bond & Lovley, 2002; van Bodegom et al., 2004; Liu et al., 2011; Zhang et al., 2012, 2013). The methanogens able to reduce iron oxides were Methanosarcina barkeri (Bond & Lovley, 2002; van Bodegom et al., 2004; Liu et al., 2011), Methanosarcina voltaei (Bond & Lovley, 2002), Methanosarcina mazei (Zhang et al., 2012), and Methanothermobacter thermotogae (Zhang et al., 2013).

The iron oxides used in these experiments were amorphous iron (Bond & Lovley, 2002; van Bodegom et al., 2004), ferric iron in non-tronite (Liu et al., 2011; Zhang et al., 2013), illie–smectite (Zhang et al., 2012), and montmorillonite (Zhang et al., 2013). On the other hand, some studies showed the possible role of ferric iron-oxide minerals as natural electrochemical devices to create unique syntrophic interactions facilitating methane production (Kato et al., 2012; Jiang et al., 2013). The previous studies, such as that of Bond & Lovley (2002), focused on this ability of methanogens to reduce iron and used high concentrations of electron donors such as hydrogen and anthraquinone-2,6-disulphonate (AQDS) as the electron shuttle.

In this work, we explored the direct coupling between methanogenesis and iron reduction by methanogens by testing the shift from hydrogenotrophic methanogenesis to iron-oxide reduction in conditions that mimic as close as possible the natural ones. This was achieved by growing *M. barkeri* with minimum possible yeast extract as organic amendment, and mainly in an N2/CO2 atmosphere. The CO2 was added to maintain the carbonate system and the pH close to the natural condition, and as a potential carbon source for autotrophic processes. Poorly crystalline iron [Fe(III)(OH3)] in different concentrations was then added as were potential abundant electron shuttles AQDS and phenaazine-1-carboxylate (PCA). AQDS has been used as an analogue for humic acid electron shuttling (e.g., Lovley et al., 1996; Newman & Kolter, 2000), and PCA has been used as a hydrophilic analogue of the methanophenazines (electron carriers in some archaeal membranes) with known abilities to reduce ferric oxides (Wang & Newman, 2008). PCA is a more relevant addition than AQDS, as quinones (and AQDS as their analogue) have not been found in methanogens, but phenazine compounds were found (and PCA is their analogue), and they have a quinone-like role in electron transfer (Wang & Newman, 2008). Isotopically labeled CO2 was added to explore the carbon source for methanogen growth. Our results shed light on the possible biogeochemical role of ferric oxide minerals in deep sediments.

**METHODS**

**Experimental design**

Three sets of experiments were performed in this study with pure cultures of *M. barkeri*, each of them 12–14 days (I—March 2013, II—April 2013, III—May 2013). *Methanosarcina barkeri* (MS), DSM 800, was obtained from Deutsche Sammlung von Mikroorganism und Zellkulturen (DSMZ, Braunschweig, Germany). Cultures were grown at 37 °C and 200 rpm in 125 mL crimp-top bottles using a modified Hungate technique (Hungate, 1969) with 50 mL modified (see below) phosphate buffer medium for methanogens (Boone et al., 1989), 0.5 mL vitamin solution (DSMZ medium 141), without resazurin (its blue-purple color interferes spectrophotometrically with the fixed Fe(II)-ferrozine compounds), and with 0.5 mL of reducing agent. Three different reducing agents (RAs) were used: sodium sulfide solution (final concentration of 1 mM), titanium-citrate (final concentration of 2.56 mM) (Zehnder & Wurhmann, 1976; Jones & Pickard, 1980), and titanium-nitrilotriacetate (Ti(III)-NTA) (final concentration of 2.56 mM) (Moench & Zeikus, 1983). All media were prepared anaerobically and sterilized. The recipes for the media and the reducing agents are detailed in Appendix 1. Bottles were inoculated with 1 mL of an exponential phase *M. barkeri* culture. H2/CO2 (80:20) was used as the energy source, as it was found to be the optimal electron donor for the growth of these methanogens (Boone et al., 1989) and has thus become the standard substrate. It was replenished every few days to restore the initial ~100 KPa overpressure within the bottles to encourage further growth. The design for all experiments is provided in Table A1 in Appendix 2. Most were repeated in triplicate under N2/CO2 atmosphere and with the addition of poorly crystalline ferrihydrite. In the first experiment, 14 pure cultures were grown for approximately 2 weeks in the methanogens’ medium but with only 2 g of organic material, 1 g of yeast extract and 1 g of tryptica peptones, and with titanium-citrate as the reducing agent. Four bottles were then killed via autoclave (121 °C, 20 min). All bottles were then purged twice (5 min each time) with N2/CO2 (80/20) and then 12 bottles (including killed bottles) with CH4/CO2. It should be noted that in general, this way of purging is sufficient to change the atmosphere in the incubation bottles. The methanogens would not have grown if there had been any oxygen left in the bottle. Also, the added reducing agent will scrub out any residual oxygen in the bottle that the flushing did not purge. We added CO2 to keep the system as close as possible to the natural condition and its pH. Some bottles were purged with CH4 to check whether there is also use of the methane itself (AOM by reverse
methanogenesis); however, in this time scale, this process
was not evident. One milliliter of AQDS (5 mM) was
added to five bottles for a final concentration of 0.1 mm
(two autoclaved and three non), and 1 mL of poorly crys-
talline ferrihydrite (0.5 M) for final concentration of 10 mm
was added to all but two bottles. Poorly crystalline ferrihy-
dride was prepared by titrating FeCl₃ with 10 N NaOH to
pH 7. To the other two bottles, 1 mL of 1.5 M NaCl was
added instead for the same final concentration of Cl⁻ of
30 mM. Ferrous iron was measured in the beginning of the
experiment and after 1, 3, 6, 9, and 12 days.

Based on the first experiment (shown in the results), it
was clear that the H₂ in the headspace was not the primary
electron donor, and a second series of experiments was
designed to explore what chemical was being used as an
electron donor in the medium. We used the same medium
but with minimum organic supplementation that still
allowed the methanogens to grow (25% of the original 2 g
of the yeast and trypticase peptones—0.5 g each). When
the methanogens reached the exponential stage with H₂/
CO₂ in the headspace, we switched to N₂/CO₂ atmo-
sphere (purging three times for 5 min each), autoclaved
four bottles and started the experiment. Two milliliter of
methane were added to two non-killed bottles and to one
killed bottle. 1.5 mL of 250 mM poorly crystalline ferrihy-
dride was added to all bottles to reach final concentrations
of 7.5 mM. We also added 1 mL of 5 mM PCA to three
bottles (one killed and two non-killed) to reach a concen-
tration of 0.1 mM [according to Wang & Newman
(2008)]. Ferrous iron and methane were measured across
the 12 days of the experiment.

In the third experiment, we attempted to identify the
electron donor and explore the carbon source for the
methane production. Therefore, we used the same medium
with only 25% of the normal amount of organic supple-
mentation, with titanium-citrate or Ti-NTA (final concen-
trations of 2.56 mM) as the reducing agents, and with
poorly crystalline ferrihydrite (7.5 mM final concentration).
We added 0.5 mL labeled (13C) HCO₃⁻ to seven bottles
for a final concentration of 3 mM (typical concentration for
aquatic systems). We also used sodium sulfide and sulfide-
citrate as reducing agents to test the methane production
in the absence of amorphous iron, as the redox coupling
between sulfide and oxidized ferric iron is well known, will
lead to iron sulfide precipitation, and will not eliminate the
option of Ti(III) as an electron donor. Ferrous iron, methane, and δ¹³CCH₄ were measured during the 14 days
of the experiment. In all experiments, pH was monitored
to verify that it remained circum neutral.

Analytical methods

Methane and δ¹³CCH₄ were measured from the
headspace as described in Kinnaman et al. (2007). Methane concentrations were measured by a gas chroma-
matograph (GC) using a Shimadzu GC-14A equipped with
a flame ionization detector (FID) and a packed column
(n-octane on Res-Sil C) with error of 3%. The δ¹³CCH₄ was
measured via continuous flow isotope ratio mass spectrom-
etry (IRMS) coupled with a trace GC via a GC/Combustion
III interface (Thermo). The precision of the measurement was ±0.3‰ and the results are reported relative
to the Vienna Pee Dee Belemnite (VPDB) standard.

One milliliter of the solution was sampled for ferrous iron
concentrations. Ferrous iron was fixed immediately using
the ferrozine method (Stookey, 1970), and the absorbance
at 562 nm was measured on a spectrophotometer with an
error of <7 μM.

RESULTS

In the first experiment, M. barkeri was grown in the pres-
ence of 10 mM amorphous iron oxide, with titanium-
citrate (Ti(III)-cit) as the reducing agent, N₂/CO₂ or
CH₄/CO₂ headspace, and with and without 0.1 mM
AQDS. Throughout the experiment, the pH remained
neutral in all bottles. Ferrous iron concentration increased
dramatically through this experiment (Fig. 1), indicating
intensive biological reduction of iron oxides by the metha-
nogens; 1000 μM of ferrous iron was produced over the
13 days of the experiment. In the first day, there was also
abiotic reduction of iron in the killed bottle containing the
AQDS treatment. The biological reduction occurred in
bottles with both N₂/CO₂ and CH₄/CO₂ headspace.

Besides the AQDS addition that caused a significant abiotic
reaction during the first day, the other experiments showed
the main increase in the following days, and iron reduction
in the CH₄/CO₂ headspace was similar in the bottles with
and without AQDS addition in the end of the experiment,
reaching the same ferrous iron levels by the end of the
experiment (13 days). The highest rates of iron reduction

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were in the N₂/CO₂ atmosphere, indicating that the main electron donor to this redox reaction was present in solution and was not in the headspace. The possible electron donors were compounds present initially in the medium, biosynthetic products that accumulated during growth, and the Ti(III)-citrate (either Ti(III) or citrate).

Black particles precipitated during the experiment in all non-killed bottles treated with poorly crystalline ferrihydrite, while the killed samples retained their original orange-brown color (Fig. 2). Considering the possible electron donors, the black precipitated minerals could be an early stage of magnetite (Fe₃O₄), as already observed in dissimilatory iron reduction experiments (Lovley et al., 1987), or ilmenite (Fe(III)Ti(IV)O₄). Using scanning electron microscopy (SEM), we observed relatively large (~100 µm) particles containing both iron and titanium (Fig. 3). It should be noted that the Ti in the reducing agent is Ti(III), which is a dissolved species, whereas Ti (IV) is the particulate Ti in the possible minerals. Thus, to obtain Ti in the minerals, it needs to be in the form of Ti (IV), unless the whole reducing agent was adsorbed and detected as part of the mineral. However, as the solids were still amorphous, we were not able to determine whether the titanium and iron formed one compound or were part of a mixture. Other particles that were detected by SEM were NaCl, iron oxides, and small particles of Fe-PO₄ with fabric texture (possibly a stage of vivianite [Fe (II)₃(PO₄)₂]). The particles were also examined by x-ray diffraction (XRD), but results were inconclusive because of the amorphous characteristics of the particles. The biological role of titanium has yet to be determined, but a role for the particles as an early stage of magnetite, based on the SEM results, color and its previous observation in dissimilatory iron reduction (Lovley et al., 1987), is certainly reasonable. It should be noted, however, that the particles were not attracted to a magnet as found in Lovley et al. (1987).

The second experiment focused on the electron donor in the solution and the reaction mechanism. Therefore, the headspace atmosphere was N₂/CO₂ (80/20) in all bottles. As we did not successfully grow the methanogens in the absence of organic supplementation (and the possible electron donors within), we grew them with limited concentrations of yeast extract and trypticase peptones. For the reaction mechanism, as the AQDS did not increase iron reduction, it seems unlikely that electron shuttling through small, humic acid-like molecules as described by Newman & Kolter (2000) occurred in these experiments. Therefore, we added PCA as a hydrophilic version of the methanophenazines (Wang & Newman, 2008). We also lowered the concentration of ferric iron to 7.5 mM from 10 mM.

The pH remained near neutral throughout the experiment. Again, microbial iron reduction was intensive with the N₂/CO₂ atmosphere (Fig. 4A), indicating that the main electron donors were not in the headspace. The addi-
tion of methane did not change the ferrous iron production. However, addition of 0.1 mM PCA increased the reduction by ~20%, suggesting that methanophenazines were involved in iron reduction. Small amounts of methane were produced in all the non-killed samples, reaching maximum values of ~0.1% in the bottles with the PCA addition (Fig. 4B).

The reaction order was calculated by plotting the ferrous iron increase in the first 6 days vs. the poorly crystalline ferrihydrite addition. The obtained linear relationship fits a first order reaction where ferric iron is reduced to ferrous iron (equation 1):

$$\frac{d[\text{Fe(II)}]}{dt} = k[\text{Fe(III)}]$$  \hspace{1cm} (1)

where $k$ is the reaction rate coefficient. In this case (equation 2),

$$[\text{Fe(II)}]_t = [\text{Fe(III)}]_0 \times (1 - e^{-kt})$$  \hspace{1cm} (2)

and a straight line is obtained by plotting ferrous concentrations vs. the initial ferric iron concentrations, as was indeed observed (Fig. 5). The calculated rate coefficient was 0.015 day$^{-1}$. At the end of the experiments at least 10% of the added Fe(III) was converted to Fe(II). We did not measure the growth rate of the methanogens, because of interference from the black precipitate that formed.

The third experiment was conducted with N$_2$/CO$_2$ (80/20) in the headspace and focused on the electron donor in the solution, the carbon source for methanogenesis, and whether the iron reduction competes with methanogenesis. The results of this experiment show that in both media, with Ti-NTA and Ti(III)-citrate, there was biological reduction of iron (Fig. 6A), indicating clearly that citrate is not the electron donor. It should be noted that in the Ti-NTA experiment, there was also significant abiotic activity, and that after 8 days there was a drop in the ferrous iron concentrations, possibly due to precipitation. However, the methanogens seemed to recover later in the experiment. It seems, therefore, that either the Ti (III) or compounds in the yeast served as the electron donors for iron reduction. We did not attempt to conduct an experiment with iron addition and sulfide as the reducing agent, because the redox coupling between them is obvious and would not inform the involvement of Ti(III).

We did inoculate bottles with sodium sulfide as the reducing agent without Fe(III) addition and found that methane was produced (5% of the headspace). This indicates that organic compounds are sufficiently available in the yeast for redox reactions and suggests that the main electron donor can be in the yeast. Methane concentrations also increased in the Ti-citrate experiment (Fig. 6B).

Comparing the methane increase with and without amorphous iron added to the Ti-citrate bottles (Fig. 6B) indicates clearly that methane production is inhibited by iron reduction. The results show nicely that the addition of Fe(III) causes iron reduction and lesser methane production. In the Ti-NTA experiment, the addition of

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Fig. 4 Concentration changes over time of Fe(II) (A) and CH$_4$ (B). The experiments were conducted with Ti(III)-citrate as reducing agent (RA) in medium, limited amount of yeast (25%), 7.5 mM addition of amorphous Fe(III)-oxides, and N$_2$/CO$_2$ headspace (HS). Two milliliters of CH$_4$ was added to the experiment marked with diamond. The error bars are smaller than the symbols.

Fig. 5 Combined three experiments showing the concentration increase of Fe(II) after 6 days of experiment vs. the initial amorphous Fe(III)-oxides addition in all experiments with the N$_2$/CO$_2$ headspace (HS) and Ti(III)-citrate as the reducing agent. The change correlates best to a linear fit.

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amorphous iron nearly stopped methane production. It seems, however, that each system (with its certain reducing agent and addition) behaves slightly different, and thus, more iron reduction in one system will not necessarily lead to less methane production than in the other system. Labeled methane was produced in all the non-killed bottles from labeled CO₂ (Fig. 6C) with the highest carbon isotope enrichment (above 900‰) in the Ti-NTA experiment, where, there was the least amount of methane produced.

DISCUSSION

Previous studies have demonstrated the inhibition of methanogenesis under iron-reducing conditions due to competition of methanogens and iron-reducing bacteria for common substrates such as acetate and hydrogen (e.g., Lovley & Phillips, 1986; Roden & Wetzel, 1996; Conrad, 1999; Roden, 2003). Direct iron reduction by methanogens has also been demonstrated previously with electron donors such as hydrogen and acetate (Bond & Lovley, 2002; van Bodegom et al., 2004; Liu et al., 2011; Zhang et al., 2012, 2013). Here, we show the following: (i) a fast transition to iron reduction in conditions that resemble as closely as possible the natural ones with limited substrate, a nitrogen headspace, and no addition of hydrogen or acetate; (ii) the reaction rate order of the process and a clue on the electron transport, using different concentrations of iron and electron shuttles that may be relevant to archaea such as PCA; and (iii) a link between methanogenesis and iron reduction in the environment in the methanogenic zone of marine and freshwater sediments. Further study is needed to identify clearly the electron donor and the exact electron transport mechanism.

Our results show that the methanogen *M. barkeri* can reduce iron and produce methane simultaneously and even switch instantly from methanogenesis to iron reduction over the course of an experiment where methane production was inhibited. The methanogens started to reduce iron immediately after the addition of iron oxides (on the first day) without pre-incubation with ferric iron, with limited substrate, and without H₂ in the headspace. The clear observation of the rapid shift to iron reduction close to natural conditions with nitrogen atmosphere and limited substrate was reproduced in all treated non-killed bottles in the three experiments. Moreover, the rate was consistently higher with larger additions of iron in the same nitrogen headspace. Methanogenesis slowed significantly when reactive iron was present. It was not totally ceased otherwise ¹³C labeled methane would not have been generated from added ¹³C labeled CO₂. However, there was much less methane production (~20% in the case of Ti-cit and less than 5% with Ti-NTA) at the termination of the experiment with the addition of Fe(III) as compared to the experiments without its addition (Fig. 6). This result indicates that at least 80% of the methane production was inhibited by the Fe(III) reduction. Thus, Fe(III) here inhibits methanogenesis and clearly does not serve as a redox shuttle stimulating methane production.

The results raise several questions. What are the required conditions for iron reduction coupled to methane production, and what is the reaction order? What is the electron transport mechanism? What are the electron donors for this transition from methane production to iron reduction? Is it possible that the methane itself is the electron donor, thus creating iron-driven methane oxidation in a single organism?
Reaction rate order

Dissimilatory iron-oxide reduction plays an important role in the reductive dissolution of the poorly soluble iron oxides. Factors impacting the rate of reduction of iron oxides include crystallinity, particle size, available surface area, the presence of chelating agents, and electron shuttles such as cysteine, humic acids, and extracellular quinones (Lovley et al., 1996; Newman & Kolter, 2000; Doong & Schink, 2002). In our experiments we used highly reactive iron oxides, and we conclude that the methanogens, when fed iron oxides, ceased almost all of the methane production and instead funneled electrons to reduce iron.

Iron reduction was found to be a pseudo first order reaction. This means that it was dependent only on the initial iron concentrations, as expected for enzymatic processes with no substrate limitation. Our results are similar to the results of Bonneville et al. (2004), who showed that bacterial iron reduction exhibits a Michaelis–Menten kinetic dependence with respect to the concentrations of the oxidized iron substrate. Our rate coefficient was 0.015 day\(^{-1}\) and provides a minimum estimation for the rate of iron reduction, as part of the ferrous iron produced during iron reduction precipitated from solution (Fig. 2). This rate coefficient is higher than was observed, for example, for iron-reducing bacteria and ferrihydrite \(0.0012\) day\(^{-1}\) with 0.5 mM cysteine, Doong & Schink (2002). This high rate coefficient may indicate that the enzymatic kinetics of iron reduction by methanogens are elevated compared to some iron-reducing bacteria. The enzyme kinetics seemed to be affected by PCA addition; the reactivity of the iron oxide increased when PCA was added to the experiment. This is not surprising, because PCA has been shown to serve as an electron shuttle with an appropriate redox potential (see explanation below) (Wang & Newman, 2008).

The electron transport mechanism

Microbial respiration of ferric oxide minerals coupled to the oxidation of organic carbon has been investigated intensively. It is understood from previous works that ferric iron oxides can serve as either the terminal electron acceptors (in dissimilatory iron reduction) or as electron shuttles like nanowires (e.g., Kato et al., 2012; Jiang et al., 2013).

When nanosized iron-oxide minerals are used as electron shuttles for respiration, the suggested electron flow is via redox iron cycling (Fe(III) mineral—Fe(II) liquid—Fe(III) mineral) and transformation of iron minerals to secondary nanominerals (Jiang et al., 2013). In that case, iron cycling facilitated methane production via acetate oxidation coupled to hydrogenotrophic methanogenesis. The rate of the process presumably depends on mineralogical properties.

The ability of bacteria to use the poorly soluble iron oxides as terminal electron acceptors for respiration has also previously been demonstrated. As mentioned, several electron transport strategies for extracellular respiration with minerals have evolved including cysteine (Doong & Schink, 2002) and humic acids (Lovley et al., 1996) as electron shuttles that are reduced by the cell and then are oxidized by the iron oxides (with redox potential within the range of the reaction, see equations below). It was shown that compounds carrying quinone moieties, such as humic acids or AQDS, can effectively stimulate the dissolution of iron oxides in the presence of iron-reducing bacteria (Newman & Kolter, 2000).

Humic substances and their analogue, AQDS, are exogenous electron shuttles for iron-oxide reduction (with the quinones as the redox-active groups), where phenazines are endogenous molecules (Wang & Newman, 2008). In our experiments, AQDS did not increase iron-oxide reduction. These results are expected and consistent with the results of Bond & Lovley (2002). They showed that AQDS increased Fe(III) reduction significantly only when the headspace was H\(_2\). They also showed that H\(_2\) was the only headspace composition where AQDS could be significantly reduced. They concluded that when H\(_2\) is used as the electron donor, the reduction of Fe(III) and extracellular quinones (and AQDS as an analogue) are linked to H\(_2\) oxidation, rather than to methanogenesis, where there is electron flow from H\(_2\) to Fe(III) by methanogens directly or through the reduction of AQDS.

However, when we added PCA phenazine (which was not tested before on iron reduction by methanogens) as the hydrophilic version of the methanophenazines (Wang & Newman, 2008), ferrous production increased by 11–28%. It is clear that PCA serves as shuttle in the reduction of Fe(III) to Fe(II), as the addition of PCA increased the Fe(III) reduction rates. The results suggest that methanophenazines, phenazine derivatives that are formed in membranes of methanogenic archaeca (Beifuss et al., 2000), might be involved in the electron transport under natural conditions when H\(_2\) is limited or absent. This is reasonable, as methanogens lack the usual quinones, but methanophenazines were found to have a similar role to quinones in electron transport (Beifuss et al., 2000; Tietze et al., 2003). As quinones, their ability to exist in both an oxidized and reduced form enables them to participate in the electron transport chain. Methanophenazines were shown to function as electron carriers in the cytoplasmic membrane (mediating the electron transport between the membrane bound enzymes) and to take part in the energy-conserving electron transport (Beifuss et al., 2000).

The electron donor for iron reduction

The microbially-mediated reduction of iron oxides occurred both in N\(_2\)/CO\(_2\) and CH\(_4\)/CO\(_2\) atmospheres. The highest rates of iron reduction were found in the N\(_2\)/
CO₂ atmosphere, indicating that the main electron donor to this redox reaction was in the solution, as neither N₂ nor CO₂ can be electron donors. Methane oxidation cannot be ruled out; however, methane could not be a significant electron donor, because the intensive reduction occurred also in its absence. These results are expected, considering the slow rates of AOM and its time scale (e.g. Sivan et al., 2014). Methane addition seems to even inhibit iron reduction slightly (Fig. 1), which is not surprising as high levels of methane were already shown to inhibit the methanogen’s activity in some pathways of methanogenesis, consistent with thermodynamic considerations (Beer & Blodau, 2007).

The possible electron donors in the solution were compounds in the yeast, trypticase, accumulated metabolites, and Ti(III)-citrate (either Ti(III) or citrate). Citrate is not known to act as an electron donor, and indeed our experiment with Ti-NTA, which showed intensive iron reduction, confirmed that citrate was not responsible for reducing iron oxides (Fig. 6). This leaves the organics or the Ti(III) as the possible electron donors. As the organics in the yeast are assumed to not be used as a catabolic substrate, Ti(III) is the probable electron donor candidate. Thermodynamically, the reduction of iron by titanium oxidation is indeed possible and would yield energy, considering the redox potentials [approximately −50 mV for amorphous Fe(OH)₃ and −300 mV for Ti(IV)-cit (Siburt et al., 2010) or approximately −350 mV (Jones & Pickard, 1980)], and that titanium concentrations were sufficient to sustain this redox reaction (2.56 mM compared to −1 mM increase of Fe(II) and 1:1 redox ratio). However, titanium has not yet been shown in any study to participate in biological reactions (Buettnner & Valentine, 2012), and we failed to prove its involvement here based on these experiments because the methanogen cultures would not grow without yeast extract. It seems, therefore, that we cannot rule out one of the explanations for our results, that the electron donor for iron-oxide reduction is contained within the yeast. The electron can be donated directly from one of the added organic compounds or from hydrogen that is produced in the system. It has been shown (Valentine et al., 2000) that M. barkeri can produce hydrogen from complex compounds under low hydrogen conditions and that the hydrogen production is likely mediated by the hydrogenase system. Based on these observations, the relevant possible half redox processes and their half potentials (vs. standard hydrogen electrode (SHE) at pH 7) are as follows:

Fe(OH)₃(am) → Fe²⁺: \( E^0 = −50 \text{ mV} \) (Wiedemeier et al., 1999)  
PCA(ox) → PCA(red): \( E^0 = −116 \text{ mV} \) (Wang & Newman, 2008)  
Methanophenazine (ox) → Methanophenazine (red): \( E^0 = −165 \text{ mV} \) (Tietze et al., 2003)  

AQDS (ox) → AQDS (red): \( E^0 = −184 \text{ mV} \) (Wolf et al., 2009)  
\( \text{CO}_2 + 8\text{H}^+ → \text{CH}_4 + 2\text{H}_2\text{O} \): \( E^0 = −240 \text{ mV} \) (Drake et al., 2006)  
\( \text{Ti(IV)} + \text{cit} → \text{Ti(III)}-\text{cit} \): \( E^0 = −300 \text{ mV} \) (Siburt et al., 2010)  
\( 2\text{H}^+ → \text{H}_2 \): \( E^0 = −414 \text{ mV} \) (Weber et al., 2006)

It can be seen that the redox potentials of the suggested electron shuttles are indeed within their expected values in this system, between the relevant possible electron donors and iron oxides. The possible reactions in the case that Ti(III) is the electron donor are:

\( \text{Ti(III)}-\text{cit} + \text{CO}_2 + 8\text{H}^+ → \text{CH}_4 + 2\text{H}_2\text{O} + \text{Ti(IV)} + \text{cit} \)
\( \Delta E = −240 + 300 \text{ mV} \)

\( \text{Ti(III)}-\text{cit} + \text{PCA(ox)} → \text{PCA(red)} + \text{Ti(IV)} + \text{cit} \)
\( \Delta E = −116 + 300 \text{ mV} \)

\( \text{PCA(red)} + \text{Fe(OH)}_3(\text{am}) → \text{Fe}^{2+} + \text{PCA(ox)} \)
\( \Delta E = −50 + 116 \text{ mV} \)

All of these reactions are exergonic and feasible. Labeled methane was produced in all the non-killed bottles from labeled CO₂ with highest carbon isotopes achieved in the Ti-NTA experiment, where there was least methane production: About 4 μmol of methane in the headspace (75 μl) with δ¹³C of almost 1000‰ of 14 days with Ti-NTA and Fe(III) addition as compared for example with 92 μmol of methane in the headspace (1800 μl) with values of only 50‰ that was produced with Ti-cit without Fe(III) addition (Fig. 6). The reason is probably a different mechanism. It seems that with the addition of Fe(III) the carbon source for methane production was mainly CO₂, via an autotrophic process, whereas without its addition the carbon may be originated mainly from other compounds. The exact carbon source associated here remains however unclear and the role of autotrophy vs. heterotrophy should be further investigated.

Implications for pore fluid profiles in sediments

The ability of methanogens to switch from methane production to iron reduction may inform an unexplained phenomenon with global distribution, that of increasing ferrous iron concentrations in pore fluid within the deep methanogenesis zone in marine and lacustrine sediments. For example, several sediment profiles from lake and marine sediments (examples in Fig. 7) have shown high concentrations of iron oxides throughout the sediments, and porewater dissolved ferrous maxima below the sulfate minimum zone (e.g., Emerson, 1976; Nembrini et al., 1982; Wersin et al., 1991; Widerlund & Ingri, 1995). Tradition-
ally, however, it is held that they should be respired earlier within the sequence of anaerobic microbial respiration. Widerlund & Ingri (1995) discussed the dissolved Fe ‘excess’ and concluded that, similarly to Wersin et al. (1991), the dissolution of iron oxides in anoxic sediments can be a slow process probably due to ‘kinetic control on iron transformation’. It seems that the traditional concept of dissimilatory respiration order is oversimplified, because it assumes no availability limitation of the electron acceptors [as Fe(III) or Mn(IV)]. In sediments, there are complex links due most likely to availability limitations.

These profiles and others (e.g., Sivan et al., 2011) indicate that iron oxides can persist through the entirety of the sulfate zone (even hundreds of meters) and that methanogenesis and iron reduction can co-exist in the deep sediment. This is shown here for pure cultures as well as in other environments such as in a lake’s water column (Crowe et al., 2011) or in marine seeps (Sivan et al., 2014). Based on our pure culture experiments, we suggest that the iron oxides can be reactivated in the deep methanogenic zone by methanogens.

According to Lovley & Phillips (1986), the reason for the decreasing use of iron oxides for respiration coupled to organic matter oxidation seems to be related to the form of the solid iron and the nature and pace of the movement of electrons between the two solid phases (organic matter to oxidized iron). Lovley & Phillips (1986) showed that while amorphous iron oxyhydroxides are microbially readily reducible, magnetite and mixed Fe(III)-Fe(II) compounds as well as most of the other oxalate-extractable ferric iron were not available for microbial reduction. They suggested that as soon as there is production of ferrous iron from reduction of amorphous iron and precipitation of this ferrous iron, the resulting mixture of ferric and ferrous iron was persistent against further reduction. There may also be biological limitation for the movement of electrons. For example, whereas in bacterial sulfate reduction there is transformation of eight electrons from organic matter to one ion of sulfate, in complete bacterial iron reduction, the equivalent transformation of electrons will require eight ferric iron oxides.

It seems that under certain conditions, iron-oxide minerals that are presumed to be largely inactive because they were not reduced during canonical iron reduction become available for methanogens, as shown here, and are favored over methane production. A link between iron reduction and methane oxidation was previously shown (Sivan et al., 2011). It was suggested there that methanogenesis ends and iron oxides become reactive because of the high concentrations of the methane that may be more available for them than other organic compounds. We suggest here that within the zone of methane production, in the case where the AG of iron-oxide reduction and methanogenesis is very close [such as in hematite reduction (Roden, 2003)], some of these less reactive iron oxides become viable substrates due to the ability of the methanogens to produce methanophenazines that are able to shuttle electrons to the iron minerals. Thus, it may become more favorable for the methanogens to reduce iron minerals than to produce methane, thereby explaining the synchronous increase in ferrous iron and methane concentrations observed in many natural environments.

Our results also improve our understanding of the geological past of the Archean ocean and the BIF (banded iron formation). It was estimated that during the BIF formation, Fe(II) oxidation to Fe(III) in the euphotic zone occurred mostly by oxygenic or anoxygenic phototrophs (e.g.,

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**Fig. 7** Examples for porewater profiles showing dissolved iron increase at the methanogenesis zone: (A) Lake sediments—Leman, Switzerland, $S^{2-}$, and dissolved Fe in two sites (after Nembrini et al., 1982), (B) Lake sediments—Greifensee, Switzerland, sulfate, sulfide, Mn(II), and Fe(II) (after Wersin et al., 1991), (C) Marine sediments—Cascadia Margin, offshore Oregon (ODP database), Fe(tot), sulfate, and methane.

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Konhauser et al., 2005; Crowe et al., 2011). About 70% of the sinking Fe(III) oxides were reduced on the seafloor by dissimilatory bacterial iron reduction, which might have also been affected by methanogenesis and methanotrophy, and the excess Fe(III) precipitated as a BIF (Konhauser et al., 2005). Our study supports the hypothesis that iron reduction, methanogenesis, and methanotrophy could coexist at the same depth (e.g., Konhauser et al., 2005; Crowe et al., 2011), competing with each other and affecting the BIF formation. Secondly, it may suggest that the Fe(III) cycling in the sea floor during the BIF formation time is even larger due to this direct reduction of Fe(III) by methanogens that use organic matter, H2, or even methane itself. On the other hand, this archaean reduction process could lead to more formations of the less reactive magnetite. At this stage, however, we cannot estimate the quantitative effect of this process on the excess Fe(III). Less methanogenesis and even iron-driven AOM could also explain the light carbon isotopic value of the precipitated carbonate phases in the BIF.

REFERENCES


**Appendix 1 Oregon Collection of Methanogens (OCM) Medium for methanogens**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH (dissolved in 100 mL H2O and bubbled with H2/CO2 (80:20) for 1 h)</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Trypticase peptones</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Mercaptoethanesulfonic acid</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Vitamin solution (see below)</td>
<td>10 mL</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>0.4 g</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgCl2·6H2O</td>
<td>1.0 g</td>
</tr>
<tr>
<td>CaCl2·2H2O</td>
<td>0.4 g</td>
</tr>
<tr>
<td>NaEDTA·2H2O</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>CoCl2·6H2O</td>
<td>1.5 mg</td>
</tr>
<tr>
<td>MnCl2·4H2O</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>FeSO4·7H2O</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>AlCl3·6H2O</td>
<td>0.4 mg</td>
</tr>
<tr>
<td>Na3WO4·2H2O</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>CuCl2·2H2O</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>NiSO4·6H2O</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>H2SeO3</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>BH3O3</td>
<td>0.4 mg</td>
</tr>
<tr>
<td>NaMoO4·2H2O</td>
<td>0.1 mg</td>
</tr>
</tbody>
</table>

Dissolved in 1 L deionized H2O (anoxic) and pH 7, autoclaved to sterilize. Vitamin solution was added after autoclaving the rest of the medium.

**Vitamin Solution (DSMZ 141):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Thiamine-HCl·2H2O</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>D-Ca-pantothenate</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>5.0 mg</td>
</tr>
</tbody>
</table>

Dissolved in 1 L deionized H2O, filter sterilized with 0.22 μm filter under anaerobic conditions.

**Sulfide stock solution (concentration of 100 mM):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2S·9H2O</td>
<td>2.4 g</td>
</tr>
</tbody>
</table>

100 mL Deionized H2O (anaerobic)

Autoclaved to sterilize.

**Titanium (III) citrate stock solution (concentration of 256 mM):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% Ti(III)Cl3</td>
<td>3.75 mL</td>
</tr>
<tr>
<td>0.2 M Na-Citrate</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Neutralized with saturated sodium carbonate to pH 7, autoclaved to sterilize.

**Titanium (III) nitrilotriacetic acid (NTA) stock solution:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q water</td>
<td>300 mL</td>
</tr>
<tr>
<td>NTA</td>
<td>9.6 g</td>
</tr>
</tbody>
</table>

9.6 mL Ti(III)Cl3 (20%) pH adjusted throughout procedure with NaOH pellets and saturated Na2CO3 solution. The solution was sparged and pressurized with N2 and then filtered through 0.22 μm filter to sterilize as it was dispersed.
**Table A1** Experimental design. Pure cultures of *Methanosarcina barkeri* were grown with 50 mL phosphate buffer medium, 0.5 mL vitamin solution, and 0.5 mL of reducing agent with the described modifications.

<table>
<thead>
<tr>
<th># Bottle</th>
<th>Red. agent</th>
<th>Yeast (g)</th>
<th>Tryptase (g)</th>
<th>Headspace</th>
<th>[Fe(III)] (mM)</th>
<th>Shuttle</th>
<th>13C-DIC</th>
<th>Autoclave</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exp. I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a,b</td>
<td>Ti(III)-cit</td>
<td>1</td>
<td>1</td>
<td>CH₄/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>2a,b</td>
<td>Ti(III)-cit</td>
<td>0.5</td>
<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
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</tr>
<tr>
<td>3a,b,c</td>
<td>Ti(III)-cit</td>
<td>0.5</td>
<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>4a,b</td>
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<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a,b</td>
<td>Ti(III)-cit</td>
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<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
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<td>X</td>
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<td>6a,b</td>
<td>Ti(III)-cit</td>
<td>0.5</td>
<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
<td></td>
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<tr>
<td><strong>Exp. II</strong></td>
<td></td>
<td></td>
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<tr>
<td>1a,b</td>
<td>Ti(III)-cit</td>
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<tr>
<td>5a,b</td>
<td>Ti(III)-cit</td>
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<tr>
<td>6a,b</td>
<td>Ti(III)-cit</td>
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<td>0.5</td>
<td>N₂/CO₂</td>
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<td>PCA</td>
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<td>X</td>
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<tr>
<td><strong>Exp. III</strong></td>
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<tr>
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<td>Ti(III)-cit</td>
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<td>0.5</td>
<td>N₂/CO₂</td>
<td>7.5</td>
<td>PCA</td>
<td></td>
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<td>0.5</td>
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<td>7.5</td>
<td>PCA</td>
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<td>Ti(III)-NTA</td>
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<td>0.5</td>
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<td>PCA</td>
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<td>X</td>
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<td>4a,b</td>
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<td>0.5</td>
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</tr>
</tbody>
</table>

AQDS, anthraquinone-2,6-disulfonate; PCA, phenazine-1-carboxylate; NTA, nitrilotriacetic acid; DIC, dissolved inorganic carbon.